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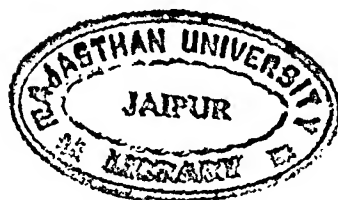
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THE RÔLE OF THE SOLUBLE SPECIFIC SUBSTANCE IN ORAL IMMUNIZATION AGAINST PNEUMOCOCCUS TYPES II AND III

By VICTOR ROSS,* Ph.D.

(From the Bureau of Laboratories, Department of Health of the City of New York, New York)

The immunization of rats against Pneumococcus Type I, by feeding the soluble specific substance has been reported in the preceding paper of this series (1). It was desirable to know whether an analogous effect could be obtained by the oral administration of the specific substance of Types II and III. This seemed probable since the conditions under which the immunity to these three types of pneumococci by oral administration of the organisms is obtained, are very similar. For example, 1 or 2 feedings of the dead bacteria from 5 cc. growth are sufficient to protect rats against any of these three types. The effect is evident in 48 hours for all of these types. Furthermore, the Berkefeld filtrates of sodium glycocholate-dissolved Types II and III pneumococci will protect against the homologous types, just as the corresponding solution of Type I does (2).

When the specific substance of Type I was fed to rats, the percentage of animals protected was, in general, smaller than when the whole organism or the dissolved cell was used, although in some of the experiments the results were so like those obtained when the bacteria are employed as to be indistinguishable from them. It was expected that a similar difference would probably exist between the effects of feeding the specific polysaccharides of Types II and III and the corresponding organisms, in the event that the former were found to confer an immunity. Such a difference, however, would make it more difficult to secure convincing evidence for these types, since the

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increased resistance which follows the ingestion of Types II and III pneumococci is not so great as it is in the case of Type I. The variation in resistance to Types II and III among normal rats, referred to in an earlier paper (2), was also expected to add to the difficulty.

The present data record the results of experiments in which the resistance of rats to Types II and III pneumococci, following the ingestion of the corresponding soluble specific substances, was measured. Several experiments in which the Type I specific substance was fed and the resistance to Types II and III was measured, and several in which the resistance to Type I was determined after feeding the polysaccharides of Types II and III, are also included.

Methods

The Soluble Specific Substance of Types II and III Pneumococcus.—Chemical analysis[1] of the Type II specific substance yielded the following figures:—C = 45.1 per cent, H = 6.4 per cent, ash = 2.3 per cent (all on dry basis), loss on heating at 100°C. = 9.7 per cent. For Type III, the results, were:—C = 42.8 per cent, H = 5.7 per cent, ash = 1.7 per cent (all on dry basis), loss on heating at 100°C. = 6.7 per cent. The Type II substance was white in color; the Type III slightly yellow. The biuret, xanthoproteic and Millon tests were negative for both substances. A concentration of 1 part of either in 3,000,000 of water could be detected by means of antisera.

For feeding, the Type II specific substance was dissolved in water; the Type III in N/10 NaOH, followed by neutralization with N/10 HCl. The solutions were mixed with either milk or cracker meal, and the rats were fed individually. The resistance of the animals to the pneumococcus was measured by injecting graded doses of the organisms intraperitoneally, in a volume of 0.2 cc. The amounts injected ranged from 10^{-8} to 10^{-5} cc. of an 18 hour blood broth culture.

Owing to the fact that the resistance of different untreated rats to Types II and III pneumococcus is so unlike, the experiments were always done with single litters, half of the litter being used for controls, the other half for experimental animals. Even this precaution does not always avoid the irregularity in deaths among controls injected with Type II. It was also found essential to use healthy rats.

Feeding the Type II Specific Substance

Experiment 1.—Each treated (E) rat received 0.5 mg. on each of 2 successive days. Test took place 2 days after the 2nd feeding. Of 12 rats in each group, 6 died among the controls and 3 among the treated rats (Table I).

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[1] Dr. Frances Krasnow kindly did these analyses. The Pregl methods were used.

Experiment 2.—Each E rat received 0.5 mg. on each of 2 successive days. Test took place 2 days after the 2nd feeding. Of 12 rats in each group, 4 died among the controls and 5 among the treated animals (Table I).

Experiment 3.—Each E rat was fed 0.5 mg. on each of 2 successive days. Test was done 3 days after the 2nd dose. Of 12 rats in each group, there were 8 deaths among the controls and 9 among the treated animals (Table I).

Experiment 4.—Each E rat was fed 1 mg. on each of 2 successive days and the test was done 3 days after the 2nd dose. There were 4 deaths among the 12 controls and 2 among the 11 treated rats (Table I).

The results of these four experiments gave no evidence for any immunity following the ingestion of 1 or 2 mg. of the soluble specific substance of Type II pneumococcus. 26 out of 48 controls and 28 out of 47 treated animals survived. The purified sample used had been prepared from an autolyzed solution of the organisms. It was decided to try the effect of feeding the specific substance prepared in a different way.

Desiccated Type II organisms were disrupted in a pebble mill. 0.1 normal HCl was added (50 cc. for the growth from 5 l.) and the mixture was shaken in a machine for 8 hours. This was allowed to settle and then was centrifuged. Normal NaOH was added till almost neutral. A heavy precipitate settles out. After centrifugation, another drop or two of alkali was added to complete the precipitation of protein An excess of alkali must be avoided in order not to redissolve this precipitate. Following centrifugation, normal NaOH was added to make n/10 and nine volumes of 95 per cent ethyl alcohol poured in. After standing overnight, the precipitate was removed by centrifugation and dissolved in 10 to 12 cc. of water. The insoluble portion was discarded. Normal HCl was added to the solution to make n/15, and after the addition of nine volumes of alcohol, the whole was left overnight in an ice box. After centrifuging, the precipitate was dissolved in 10 cc. water. Any insoluble matter present was removed by centrifugation. The solution gave a slightly positive biuret test, and judging by the intensity of the precipitin reactions which were done with serial dilutions, contained about 2 mg. of the specific substance for the desiccated material representing 1l. of culture. Five experiments were done with three such preparations.

Experiment 5.—Each E rat received the S.S.S. solution equivalent to 100 cc. growth on each of 3 successive days. Test was done 2 days after the 3rd feeding. Of 12 controls, 9 died; of 12 treated rats, 5 died (Table I).

Experiment 6.—Each E rat received the S.S.S. solution equivalent to 66 cc. growth on each of 3 successive days and the test took place 2 days after the 3rd feeding. 8 of 12 controls and 6 out of 12 treated animals died (Table I).

Experiment 7.—Each E rat was fed the S.S.S. solution equivalent to 66 cc.

TABLE I

Resistance to Pneumococcus Type II, Following Ingestion of the Soluble Specific Substance

Experiment	Dose	Litter 1				Litter 2				Litter 3			
		C		E		C		E		C		E	
		Weight	Result	Weight	Result	Weight	Result	Weight	Result	Weight	Result	Weight	Result
	cc.	gm.		gm.		gm.		gm.		gm.		gm.	
1	10^{-8}	32	S	32	2	27	S	27	S	29	S	28	S
	10^{-7}	33	S	33	S	29	2	28	S	31	S	31	S
	10^{-6}	36	7	37	S	30	2	28	S	36	2	34	S
	10^{-5}	36	2	38	2	31	2	32	2	35	S	37	S
2	10^{-8}	27	3	27	3	26	S	28	S	46	S	31	S
	10^{-7}	31	2	30	S	28	S	28	1	41	2	37	S
	10^{-6}	32	S	32	2	32	S	31	S	41	S	38	S
	10^{-5}	33	1	35	2	32	S	30	S	32	S*	46	2
3	10^{-8}	35	S	32	2	34	2	31	2	44	S	39	S
	10^{-7}	38	S	35	S	35	2	38	1	45	2	48	S
	10^{-6}	40	2	35	2	38	2	38	2	49	1	50	2
	10^{-5}	44	2	37	5	39	1	38	3	51	S	52	2
4	10^{-8}	32	S			41	S	42	S	43	S	41	S
	10^{-7}	35	S	38	S	43	S	45	S	45	S	45	S
	10^{-6}	43	S	40	S	44	S	47	S	56	2	52	S
	10^{-5}	43	2	41	S	48	2	49	2	58	2	58	2
5	10^{-8}	41	S	45	S	41	S	37	S	41	2	39	2
	10^{-7}	45	2	47	2	43	2	45	S	41	S	39	2
	10^{-6}	47	2	49	S	44	2	46	2	41	3	42	2
	10^{-5}	50	2	52	S	45	2	49	S	46	1	46	S
6	10^{-8}	38	S	36	2	49	2	44	2	54	2	55	S
	10^{-7}	47	2	45	S	52	2	52	S	55	2	57	S
	10^{-6}	48	2	46	2	57	2	54	S	54	S	60	2
	10^{-5}	49	1	50	2	65	S	63	S	72	S	62	2
7	10^{-8}	46	S	46	S	43	S	46	2	49	S	45	S
	10^{-7}	50	2	48	S	50	S	49	S	51	S	47	2
	10^{-6}	52	S	48	2	51	S	51	2	51	S	51	2
	10^{-5}	52	S	54	S	52	2	52	S	57	2	56	2

C = control. E = treated rat. S = survived. Number = died,—days.
* Became ill but recovered.

TABLE I — Concluded

Experiment	Dose	Litter 1				Litter 2				Litter 3			
		C		E		C		E		C		E	
		Weight	Result	Weight	Result	Weight	Result	Weight	Result	Weight	Result	Weight	Result
5	10.1	36	1	40		45		44	S	41	2	42	S
	10.2	29	2	45	2	46	2	47	S	47	2	44	2
	10.3	42	2	48	2	51	2	49	1	50	2	46	2
	10.4					56	2	56	S			47	2
9	10.1	36	2	37	S	42	S					26	S
	10.2	39	S	39	S	44	S			27	S	29	S
	10.3	39	1	39	S	44	2	40	2	32	S	29	S
	10.4	47	2	41	S	46	1	43	1	33	2	31	2

growth on each of 3 successive days. The animals were tested 2 days after the 3rd feeding. 3 out of 12 controls and 6 out of 12 treated rats succumbed (Table I).

Experiment 8.—Each E rat was fed S.S.S. solution equivalent to 66 cc. growth on each of 3 successive days. The test was done 2 days after the last feeding. 7 out of 11 treated animals and all 10 controls died (Table I).

Experiment 9.—Each E rat received S.S.S. solution equivalent to 66 cc. growth on each of 3 successive days. Test was done 2 days after the last dose. 6 out of 12 controls and 5 out of 11 treated rats died (Table I).

Out of an equal number of animals in the two groups (58), there were 36 deaths among controls and 29 among the treated rats. Although this shows a greater survival rate among the treated rats, the difference is too small to be regarded as related to the ingestion of the soluble specific substance.

Feeding the Type III Specific Substance

Experiment 1.—Each E rat received 0.43 mg. on each of 2 successive days. The test was done 3 days after the 2nd feeding. Of 8 treated rats, 1 survived. All 8 controls died (Table II).

Experiment 2.—Each E rat received 0.5 mg. on 1 day followed by 0.45 mg. the next day. Test was done 3 days after the 2nd dose. 3 out of 12 treated rats and 1 of 12 controls lived (Table II).

Experiment 3.—Each E rat received 0.5 mg. on each of 2 successive days. Test was done 3 days after the 2nd feeding. 8 out of 12 treated rats and 1 of 12 controls survived (Table II).

TABLE II

Resistance to Pneumococcus Type III, Following Ingestion of the Soluble Specific Substance

Experiment	Dose	Litter 1				Litter 2				Litter 3			
		C		E		C		E		C		E	
		Weight	Result	Weight	Result	Weight	Result	Weight	Result	Weight	Result	Weight	Result
	cc.	*gm.*		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*	
1	10^{-8}	51	2	51^4	2	63	2	57^7	S				
	10^{-7}	52	2	52^1	2	63	2	64^5	2				
	10^{-6}	59	2	63^2	2	64	2	68^6	2				
	10^{-5}	62	2	64^3	2	67	2	76^8	2				
2	10^{-8}	43	2	40^1	2	50	S	51^5	S	48	2	53^9	S
	10^{-7}	49	2	59^2	S	53	2	53^7	2	52	2	62^{10}	2
	10^{-6}	57	2	60^3	2	56	2	57^6	2	54	2	64^{11}	2
	10^{-5}	61	1	68^4	2	62	1	63^8	1	56	2	65^{12}	1
3	10^{-8}	39	S	37^{11}	S	42	2	43^2	S	46	2	46^3	S
	10^{-7}	45	2	41^7	S	44	1	43^9	S	47	1	47^8	S
	10^{-6}	45	1	44^{12}	S	50	1	50^{10}	S	48	1	48^6	2
	10^{-5}	51	1	49^1	1	53	1	50^1	2	58	1	52^5	2
4	10^{-8}	38	2	38^3	S	32	2	30^1	2	40	2	37^{12}	2
	10^{-7}	39	2	37^2	2	36	2	36^4	2	41	2	42^9	2
	10^{-6}	40	2	45^5	2	36	2	39^7	2	46	2	49^{10}	2
	10^{-5}	48	2	45^6	2					47	2	48^{11}	2
5	10^{-8}	46	2	45^7	S	50	S	49^3	2	54	2	45^9	S
	10^{-7}	47	2	47^6	2	51	2	52^4	2	57	2	51^2	2
	10^{-6}	47	2	47^5	2	52	2	53^1	2	59	2	54^{10}	2
	10^{-5}	49	2	51^{11}	2	60	2	55^{12}	2	64	2	58^8	2
6	10^{-8}	33	2	32	S	36	S	37	S	47	2	41	S
	10^{-7}	34	2	39	2	40	2	38	S	47	2	48	S
	10^{-6}	36	1	39	2	42	1	39	2	47	2	51	2
	10^{-5}	37	1	45	1	55	1	48	2	48	1	51	1
7	10^{-8}	25	S	19	S	26	S	25	S	25	2	22	S
	10^{-7}	27	2	27	S	27	S	29	S	28	2	28	2
	10^{-6}	27	1	27	S	27	1	29	S	28	1	29	S
	10^{-5}	30	S	28	1	30	1	30	1	30	1	29	1

The figures appearing as exponents of the weights of the E rats in the first five experiments above, identify the rats in Table II of the paper "Fate of the orally administered soluble specific substances of Pneumococcus Types I, II and III," Reference 3. Experiments 1, 2, 3, 4 and 5 above correspond to Experiments 9, 10, 11, 12 and 13, respectively, in that table.

Experiment 4.—Details same as in Experiment 3. 1 out of 11 treated rats lived. All 11 controls died (Table II).

Experiment 5.—Details same as in Experiment 3. 2 treated animals and 1 control out of 12 in each group survived (Table II).

Experiment 6.—Each F rat received 1 mg. on each of 2 successive days. Test was done 3 days after the 2nd feeding. 5 treated and 1 control animal survived (Table II).

Experiment 7.—Each E rat received 0.5 mg. on each of 2 successive days. Test was done 3 days after 2nd feeding. 9 treated rats and 5 controls survived. In the 4th litter, which does not appear in the table, of 3 controls injected with 10^{-8},

TABLE III

Resistance to Pneumococcus Type II, Following Ingestion of Type I Soluble Specific Substance

Dose	Litter 1				Litter 2				Litter 3			
	C		D		C		E		C		E	
	Weight	Result	Weight	Result	Weight	Result	Weight	Result	Weight	Result	Weight	Result
cc.	gm.		gm.		gm.		gm.		gm.		gm.	
10^{-1}	36	S	42	S	33	S	32	S	39	S	38	S
10^{-2}	39	1	45	S	33	S	34	S	44	S	46	S
10^{-4}	41	S	43	S	38	S	37	S	46	2	46	S
10^{-5}	41	S	46	1	44	S	44	2	56	1	49	2
10^{-1}	36	S	37	2	38	2	35	2	37	2	35	2
10^{-2}	39	2	39	2	43	2	39	S	38	S	36	2
10^{-4}	42	2	44	2	44	9	43	2	42	2	38	2
10^{-5}	55	1	47	2	45	1	44	2	51	2	44	1

The upper and lower parts of this table give the data of two experiments done at different times.

10^{-7} and 10^{-6} cc. respectively, the 2 latter died. Of 4 treated rats, injected with 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} cc., the last 3 died. 10^{-5} cc. might have killed a control as well if one had been injected with this dose (Table II).

Of a total of 82 control rats, 9 survived. Of 83 treated animals, 29 lived. It would seem therefore, that the ingestion of Type III specific substance was followed by an increased resistance to the homologous organism.

Since it had already been shown (2) that the immunity obtained by feeding the Types I, II and III pneumococci was type-specific, it

followed that the specific substances would act in a similar manner. Nevertheless, several experiments were done to confirm this belief. The purified soluble specific substance of Type I pneumococcus used in the experiments described in the preceding paper (1), was fed to rats which were then tested for resistance to Types II and III pneumococci. Experiments were also done to determine whether any increased resistance to Type I could be obtained by the oral administration of the specific substances of Types II and III pneumococci.

<div align="center">TABLE IV</div>

Resistance to Pneumococcus Type III, Following Ingestion of Type I Soluble Specific Substance

Dose	Litter 1				Litter 2				Litter 3			
	C		E		C		E		C		E	
	Weight	Result	Weight	Result	Weight	Result	Weight	Result	Weight	Result	Weight	Result
cc.	*gm*		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*	
10^{-8}	57	S	56	S	51	S	54	2	55	S	60	S
10^{-7}	57	2	58	S	55	2	62	2	66	S	62	2
10^{-6}	62	2	58	S	57	2	64	S	67	S	69	2
10^{-5}	69	2	59	2	69	2	70	1	68	2	75	2
10^{-8}	48	S	50	S	46	S	47	S	35	*	40	11°
10^{-7}	49	2	53	S	51	S	53	2	40	2	41	11°
10^{-6}	50	1	54	1	62	S	61	2	46	2	48	1
10^{-5}	55	1	57	2			64	S				

The upper and lower parts of this table give the data of two experiments done at different times.

* Missing, probably dead.

° Heart blood sterile.

Experiment 1.—Each E rat was fed 0.5 mg. S.S.S. Type I on each of 2 successive days. The test took place on 3rd day after the 2nd dose. 3 out of 12 rats in each group succumbed when tested with Type II pneumococcus (Table III, upper part).

Experiment 2.—Each E rat received 0.5 mg. S.S.S. Type I, on each of 2 successive days. The test was done 2 days after the 2nd dose. 10 out of 12 controls and 11 out of 12 treated rats died when tested with Type II pneumococcus (Table III, lower part).

Judging from the results of these two experiments no increased resistance to Pneumococcus Type II followed the ingestion of the specific substance of Type I.

Experiment 3. Each E rat was fed 0.5 mg. S.S.S. Type I on each of 2 successive days. The test was done 3 days after the 2nd feeding. 7 controls and 7 treated rats died out of 12 animals in each group, when injected with Type III pneumococcus (Table IV, upper part).

Experiment 4. Each E rat received 0.5 mg. S.S.S. Type I, on each of 2 successive days. Test was done 3 days after the 2nd dose. 5 out of 10 controls and 5 out of 11 treated rats died when tested with Type III pneumococcus (Table IV, lower part). The delayed deaths in the 3rd litter are counted as survivors in view of the fact that the heart blood was sterile.

TABLE V

Resistance to Pneumococcus Type I Following Ingestion of Type II Soluble Specific Substance

Dose	Part 1				Part 2			
	C		E		C		E	
	Weight	Result	Weight	Result	Weight	Result	Weight	Result
cc.	gm.		gm.		gm.		gm.	
10^{-1}	118	7	103	S	80	S	75	S
10^{-2}	125	4	118	2	85	2	78	2
	118	6	123	5	85	2	79	2
10^{-3}	135	2	124	2	93	2	85	4
	134	2	131	4	91	2	81	2
10^{-4}	152	3	135	9	92	2	87	2
	152	2	137	7	89	2	87	2
10^{-5}	156	2	140	2	98	2	92	2
	153	4	170	2	90	2	89	2

Part 1 and Part 2 of this table give the data of two experiments done at different times.

There is thus no evidence for protection against Type III pneumococcus when the soluble specific substance of Type I is fed to rats. 12 out of 22 controls and 12 out of 23 treated animals died.

Experiment 5.—Each of 9 E rats was fed 0.5 mg. purified S.S.S. of Pneumococcus Type II on each of 2 successive days and was tested with Type I pneumococcus 3 days after the 2nd dose. 8 of these, and all 9 controls died (Table V, Part 1).

Experiment 6.—Each of 9 E rats received 0.5 mg. purified S.S.S. of Type II

pneumococcus on each of 2 successive days and was tested 3 days after the 2nd feeding. Only 1 survived an injection of Type I pneumococcus. A control rat survived the same quantity (Table V, Part 2).

Experiment 7.—Each of 8 E rats was fed 0.5 mg. purified S.S.S. of Pneumococcus Type III, on each of 2 successive days and was tested 3 days after the 2nd dose for resistance to Type I pneumococcus. All the treated rats died of 1 or more fatal doses (Table VI, Part 1).

Experiment 8.—Each of 8 E rats was fed 0.5 mg. purified S.S.S. of Type III on each of 2 successive days and was tested 3 days after the 2nd feeding. A single fatal dose of Pneumococcus Type I, killed the treated animals (Table VI, Part 2).

TABLE VI

Resistance to Pneumococcus Type I, Following Ingestion of Type III Soluble Specific Substance

Dose	Part 1				Part 2			
	C		E		C		E	
	Weight	Result	Weight	Result	Weight	Result	Weight	Result
cc.	*gm.*		*gm.*		*gm.*		*gm.*	
10^{-9}	65	S	69	2	70	2	62	S
	68	2	62	3	74	S	73	2
10^{-8}	70	2	69	2	84	2	75	2
	74	2	70	2	82	S	74	3
10^{-7}	75	2	71	2	84	2	78	2
	74	S	71	1	87	2	82	2
10^{-6}	78	2	80	2	91	6	95	4
	81	2	77	2	95	2	87	2

Parts 1 and 2 of this table give the data of two experiments done at different times.

It is thus seen that no immunity against Type I pneumococcus was obtained by feeding either the Type II or III soluble specific substance.

DISCUSSION

The experiments which have been done do not present any clear evidence of an increased resistance to Pneumococcus Type II, following the ingestion of the specific substance. Out of 106 control animals, 48 survived; out of 105 treated ones, 57 lived. These rats comprised

27 litters, in 12 of which there was no difference in survival rate between controls and treated animals; in 6 the controls had a lower mortality than the experimental rats, and in 9 the reverse was the case. The numerical advantage in favor of the experimental animals is too slight to permit one to conclude that it was the result of feeding the soluble specific substance. It is of interest to compare these figures with those obtained when the organisms (both intact and dissolved) were fed. Out of a total of 73 controls, 25 survived; of an equal number of treated rats, 63 lived. In all the 19 litters employed the survival rate was greater among the experimental rats.

In the case of Type III, it appears that the resistance of rats to the pneumococcus was increased by feeding the soluble specific substance. Of 82 controls, 0 lived; out of 83 treated rats, 29 survived. Of the 20 litters used, there were 4 in which the number of survivors was alike in both groups, 1 in which it was greater among the controls and 15 in which the contrary was the case. (Litter 4 in Experiment 7 is not counted.) When the whole Type III cell and the dissolved organisms were fed the proportion of animals protected was greater. Out of a total of 96 controls, 28 lived, and of 100 treated rats 79 survived. In all 9 litters comprising a part of these tests, the survival rate was greater among the treated animals. Although the higher percentage of survivals is probably accounted for partly by the fact that, in general, larger animals were used,[2] (also reflected in a similar increase among the controls) a closer comparison of the data obtained with animals of the same weights reveals that this fact is not wholly responsible, and that there is a real difference in favor of the bacteria-feeding experiments. The same was found to be true in comparing the effects obtained with the Type I pneumococcus and its specific substance.

In view of the fact that the active constituents in all three types of pneumococci are resistant to exposure to $N/12$ HCl for 3 hours at room temperature, are soluble in bile salt solutions, pass readily through the Berkefeld filter and are resistant to the gastrointestinal enzymes of the rat, and because these characteristics are consistent with the properties of the contained polysaccharides, it seems surprising that

[2] The increased resistance to Types II and III pneumococci which appears with increasing age of the rat is discussed in Reference 2.

pneumococcus on each of 2 successive days and was tested 3 days after the 2nd feeding. Only 1 survived an injection of Type I pneumococcus. A control rat survived the same quantity (Table V, Part 2).

Experiment 7.—Each of 8 E rats was fed 0.5 mg. purified S.S.S. of Pneumococcus Type III, on each of 2 successive days and was tested 3 days after the 2nd dose for resistance to Type I pneumococcus. All the treated rats died of 1 or more fatal doses (Table VI, Part 1).

Experiment 8.—Each of 8 E rats was fed 0.5 mg. purified S.S.S. of Type III on each of 2 successive days and was tested 3 days after the 2nd feeding. A single fatal dose of Pneumococcus Type I, killed the treated animals (Table VI, Part 2).

TABLE VI

Resistance to Pneumococcus Type I, Following Ingestion of Type III Soluble Specific Substance

Dose	Part 1				Part 2			
	C		E		C		E	
	Weight	Result	Weight	Result	Weight	Result	Weight	Result
cc.	*gm.*		*gm.*		*gm.*		*gm.*	
10^{-9}	65	S	69	2	70	2	62	S
	68	2	62	3	74	S	73	2
10^{-8}	70	2	69	2	84	2	75	2
	74	2	70	2	82	S	74	3
10^{-7}	75	2	71	2	84	2	78	2
	74	S	71	1	87	2	82	2
10^{-6}	78	2	80	2	91	6	95	4
	81	2	77	2	95	2	87	2

Parts 1 and 2 of this table give the data of two experiments done at different times.

It is thus seen that no immunity against Type I pneumococcus was obtained by feeding either the Type II or III soluble specific substance.

DISCUSSION

The experiments which have been done do not present any clear evidence of an increased resistance to Pneumococcus Type II, following the ingestion of the specific substance. Out of 106 control animals, 48 survived; out of 105 treated ones, 57 lived. These rats comprised

27 litters, in 12 of which there was no difference in survival rate between controls and treated animals; in 6 the controls had a lower mortality than the experimental rats, and in 9 the reverse was the case. This numerical advantage in favor of the experimental animals is too slight to permit one to conclude that it was the result of feeding the soluble specific substance. It is of interest to compare these figures with those obtained when the organisms (both intact and dissolved) were fed. Out of a total of 71 controls, 25 survived; of an equal number of treated rats, 65 lived. In all the 19 litters employed the survival rate was greater among the experimental rats.

In the case of Type III, it appears that the resistance of rats to the pneumococcus was increased by feeding the soluble specific substance. Of 82 controls, 9 lived; out of 85 treated rats, 29 survived. Of the 20 litters used, there were 4 in which the number of survivors was alike in both groups, 1 in which it was greater among the controls and 15 in which the contrary was the case. (Litter 4 in Experiment 7 is not counted.) When the whole Type III cell and the dissolved organisms were fed the proportion of animals protected was greater. Out of a total of 96 controls, 28 lived, and of 100 treated rats 79 survived. In all 9 litters comprising a part of these tests, the survival rate was greater among the treated animals. Although the higher percentage of survivals is probably accounted for partly by the fact that, in general, larger animals were used,[2] (also reflected in a similar increase among the controls) a closer comparison of the data obtained with animals of the same weights reveals that this fact is not wholly responsible, and that there is a real difference in favor of the bacteria-feeding experiments. The same was found to be true in comparing the effects obtained with the Type I pneumococcus and its specific substance.

In view of the fact that the active constituents in all three types of pneumococci are resistant to exposure to $N/12$ HCl for 3 hours at room temperature, are soluble in bile salt solutions, pass readily through the Berkefeld filter and are resistant to the gastrointestinal enzymes of the rat, and because these characteristics are consistent with the properties of the contained polysaccharides, it seems surprising that

[2] The increased resistance to Types II and III pneumococci which appears with increasing age of the rat is discussed in Reference 2.

Types I and III of the latter should be found to possess an immunizing action and that the Type II specific substance does not exert this effect. Assuming that it is the specific polysaccharide which is the active constituent when the Type II pneumococcus is fed, the absence of antigenic activity on the part of this substance in the purified form in which it was used in the present experiments may be owing to an intramolecular rearrangement during the process of its separation from the cell. Such a change, as well as the possible presence of a second active component, was proposed to account for the quantitative difference between the immunity obtained by feeding the whole organism and that which followed the ingestion of the specific substance of Type I (1). It might also explain the similar larger proportion of animals immunized by the ingestion of the Type III pneumococcus than by feeding of its specific substance.

SUMMARY

1. When the soluble specific substance of Type II pneumococcus was fed to rats, little or no increased resistance to the organism was obtained.

2. When the specific substance of Type III pneumococcus was fed, an increased resistance to the virulent organism resulted. The percentage of animals protected is smaller than when the whole or the dissolved cell is fed.

3. When the specific polysaccharide of Type I was fed, no immunity against Type II or III was obtained, and the ingestion of the specific substances of Types II and III did not protect rats against the Type I organism.

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THE FATE OF ORALLY ADMINISTERED SOLUBLE SPECIFIC SUBSTANCE OF PNEUMOCOCCUS TYPES I, II AND III

By VICTOR ROSS,* Ph.D.

(From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, and the Bureau of Laboratories, Department of Health of the City of New York, New York)

The writer has reported elsewhere (1, 2) that the ingestion of the soluble specific substance of Pneumococcus Type I by rats is followed by an increased resistance to the homologous organism. The similarities between this effect and the one produced by feeding the whole organism are rather striking. In both cases, (a) 1 feeding is sufficient, (b) the immunity appears within 48 hours, (c) when the immunity is exhausted it can be renewed by a single feeding, (d) the action is type-specific. In general, however, the percentage of animals protected and the degree of increased resistance are greater when the organisms are fed. When the specific polysaccharide of Type III is fed, the immunizing effect is less pronounced than in the case of Type I. No clear evidence of protection following the oral administration of the specific substance of Type II was obtained.

The fact that the Type I specific substance, when orally administered, immunized white rats against the pathogenic organism from which it was isolated, created an interest in its fate after ingestion. Also, since it had been suggested in an earlier communication that the feeding of the vaccine in the very early stages of human pneumonia might have a beneficial effect on the course of the disease (based on the promptness with which an immunity is created in animals), it became necessary to learn whether any of the contained polysaccharide might enter the circulation. The appearance of this substance in the blood

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in more than traces, following the ingestion of pneumococci, would be reason for avoiding such a suggested therapeutic measure since it has been shown that the pneumococcidal action of a normal serum-leucocyte mixture is inhibited by the presence of the type-specific polysaccharide (3). The resistance of this substance to the action of trypsin made it probable that it would either be absorbed as such, or eliminated unchanged. Its immunizing action made the former alternative seem a necessary prerequisite, but its polysaccharide nature appeared to make the latter very likely. These considerations would, of course, apply to the soluble specific substances of Types II and III pneumococcus as well.

Analyses of blood, urine and feces were consequently made in order to learn something about the fate of the specific substances of these three types of pneumococci in the animal body.[1] The results obtained in the fecal analysis for Types I and II, and in the preliminary blood and urine examinations for Type I, have been briefly reported elsewhere (4). The work has since been extended to include (1) analyses of the feces following ingestion of the specific substance of Type III and (2) analyses (with the aid of a more refined technique than that used at the beginning) of the blood and urine of rats fed the polysaccharides of Types I, II and III. The present paper gives a detailed description of the methods employed and the experimental data obtained.

Methods

Feeding.—The soluble specific substance of Type I was dissolved either in N/15 HCl or in Sörensen's phosphate buffer solution of pH 7.7, that of Type II in water, and of Type III in N/10 NaOH followed by neutralization with N/10 HCl. The amount administered varied from 0.25 mg. to 6 mg., but in most of the experiments was 1 mg. The desired quantity, generally in a volume of 0.2 to 0.5 cc., was placed in a small dish having a slight concavity to avoid spreading of the fluid, and cracker meal was added to make a pasty mixture. In many cases milk was substituted for cracker meal. The animals were fasted for 8 to 18 hours before feeding and only an occasional one failed to eat the material almost completely in a few minutes. Food was again given 2 or 3 hours later and consisted of bread, water, milk and lettuce.

Cages for Collection of Feces.—The rats were kept in individual cages having two

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[1] The results of the chemical analyses of these three substances are given in References 2 and 5.

graduated centrifuge tube and normal HCl added to bring the volume to the 35 cc. mark. After standing overnight, the pieces were broken up, acid added to the 40 cc. mark, the whole stirred well, allowed to stand for 1 hour and then centrifuged for 1 hour at about 2000 R.P.M. Some of the supernatant fluid was transferred to a 15 cc. centrifuge tube for further clarification, after which 3 cc. of the new supernatant fluid were removed to another 15 cc. centrifuge tube, a drop of phenolphthalein solution put in and 10 per cent solution of NaOH added until distinctly alkaline. At this stage a voluminous precipitate appeared. This was allowed to stand for 1 hour. Enough water was added to make the volume 5 cc., and the whole stirred and centrifuged. The supernatant was generally clear. 4 cc. were transferred to another centrifuge tube and N/5 or N/10 HCl was added until the color disappeared and the solution had become very slightly acid. The liquid was then made up to 4.8 cc. with water, and the whole stirred and left in an ice box overnight. Occasionally a slight sediment formed; this was removed by centrifugation. Most samples remained clear. The concentration of the soluble specific substance was now one-half that in the fluid portion of the original 40 cc. to which the feces and HCl were made up. When 0.5 mg. was fed this concentration would be 1 part in 160,000,[2] assuming complete elimination in the feces and a uniform distribution of the S.S.S. in the fecal and fluid portions of the original mixture. To the collection of feces of the control rats was added a quantity of polysaccharide equal to that fed, and the extraction was carried out simultaneously with that of the feces of the experimental animals.

Serial dilutions were made with 0.9 per cent salt solution; 0.5 cc. of each dilution was mixed with 0.5 cc. of a 1:5 dilution of antiserum corresponding to the type of the polysaccharide fed. Antiserum of another type was mixed with the extract and with one of its serial dilutions to observe any foreign sediment which might form. These mixtures were left in an ice box for 48 hours in the early experiments. This was subsequently reduced to 24 hours. The tubes were shaken and an arbitrary value of 10 assigned to the precipitates in the tubes representing the control rats, to the feces of which polysaccharide had been added. The tubes representing the fed rats were given numerical values based on this value. If 2 controls were used, the precipitates for the 2nd one were assigned values in terms of the 1st. There were thus a series of numbers for each rat equal to the number of dilutions of the extract which had been set up with serum. These numbers were averaged to obtain a final value representing the amount eliminated in the feces of each rat. It was considered that a more accurate result would be secured by setting up a series of dilutions for each extract than if only the undiluted extract were examined. The obvious difficulties associated with the extraction of such small quantities of material from feces, and the errors inherent in the method as a whole, resulted in considerable variation in the values obtained for different rats in a single experiment, as well as for the individual tubes representing each animal.

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[2] Or less, in those experiments where the original volume was greater than 40 cc.

There was ... variation in the procedure described. e.g., where the quantity of feces for one rat in an experiment was large, 3 extractions were made with HCl, and the fluid was made up to 100 cc. These are mentioned in the description of the experiment.

The resistance of rats fed Types I and II specific substances to the homologous organisms was tested, and is recorded either in this paper or in Reference 2 or 5.

RESULTS

Blood.—In those experiments in which the incubation of the serum took place at 37°C., quantities up to 6 mg. were fed, and in no case was a positive test obtained, thus showing that if present at all in the serum, the polysaccharide was there in a concentration of less than 1 part in a million. Only Type I was fed in such large quantities and tested for at this temperature of incubation. Subsequently, as described above, incubation took place in an ice box, and the tests were extended to include Types I, II and III. 1 mg. of each was fed to each rat and the animals were bled from the heart once, and then sacrificed. Blood was drawn 2, 3, 4, 5 and 6 hours after the ingestion of the polysaccharide, which took place after an 18 hour fast. A total of 90 rats (30 for each type) was examined and no evidence for the presence of the specific substance in the serum was found. This indicates that, if present at all, the concentration must have been less than 1 part in 1 or 2 millions of serum, up to 6 hours after the feeding.

Urine.—The quantity of urine, including acid, collected from the plates for any one period, varied between 3 and 10 cc., the variation having been due to different degrees of evaporation and to differences in length of the periods. This means that from 0.001 to 0.003 mg. of the specific substance would have to be present in the urine before any could be detected. Since 1 mg. was fed, it would also follow that if between 0.1 per cent and 0.3 per cent of the amount fed were present in any one sample of urine, it could be detected. If spread over 2 or

[2] It was found that the ratio of the volume of supernatant HCl to that of the fecal sediment after centrifuging should be between 3:1 and 4:1. A smaller ratio appears in some way to inhibit the final precipitation, and a larger one takes out much pigment.

more samples, it would escape detection.   Somewhat larger quantities than this might also escape detection if distributed over several collections of urine.

It was also found that 0.01 mg. is the smallest quantity of the polysaccharide of either Type I, II or III, which, when injected subcutaneously or intraperitoneally into a rat, can be detected in the urine.   Such an injection is followed by the appearance of from 0.001

TABLE I

Results of Urine Examinations Following Ingestion of Soluble Specific Substance of Pneumococcus Types I, II and III

Hours after ingestion...........	0–18			18–24			24–42			42–48			48–66			66–72		
Type S.S.S. fed...	1	2	3	1	.2	3	1	2	3	1	2	3	1	2	3	1	2	3
Rat No.																		
1	−	−	−	−	°	−	−		−	−	−		−	*		−		
2	−	−	−	−	°	−	−		−	−	−		−	−		−		
3	−	−	+	−	−	−	−	−	−	−		−	−		−	−		−
4	*	−	−	−	−	−	−	*	−	−		−	−		−	−		−
5	−	−	−	−	−	−	*	−	−	−	−	−		−	−		−	−
6	−	−	−	−	−	−	−	−	−	−	−	−		−	−		−	−
7	−	−	−	−	−	−	−	−	−		−							
8	−	−	−	−	−	−	−	−	−		−							
9	−	−	−	−	−	°	*	−			−			−		−	−	
10	−	*	−	−	−	°	*	−			−			−		−	−	
11	−			−			−			−			−					
12	−			−			−			−			−					

− = absent, + = present.
° Sample of 18 to 42 hours, negative.
* Feces present; sample discarded.

to 0.003 mg. in the urine secreted in the first 18 hours.[4]   After this time none can be found, so that one may conclude that the remainder is probably excreted at so slow a rate as to yield amounts beyond the limits of the test.

Of the urines of 32 rats fed 1 mg. each of either Types I, II or III specific substance, only 1 urine gave a positive test.   This was urine

[4] No attempt was made to learn whether the urine of less than 18 hours had any.

of Rat 3, fed Type III (Table I). The 18 hour sample gave a faintly positive test. ... next sample gave a negative result. Although no forms were observed in this sample, it is possible that a minute quantity was present and accounted for the positive precipitin test.

It appears therefore that less than 0.1 per cent to 0.3 per cent of the amount ingested was present in any one urine up to 72 hours, and that

TABLE II

Per Cent of Orally Administered Soluble Specific Substance of Pneumococcus Types I, II and III Found in the Feces

	Type I				Type II					Type III			
Exp. No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Control A	100	100	100	100	100	100	100	100	100	100	100	100	100
Control B		93		53		100	102	93	85	133	80	88	113
Rat No.													
1	62	50	90	93	73	95	74	80	45	75	80	22	93
2	62	59	103	—	95	103	91	90	55	30	—	34	70
3	71	56	110	56	85	95	63	77	63	33	90	24	78
4	46	80	80	84	78	80	79	97	85	40	—	—	72
5	59	75	80	67			90	70	43	77	107	30	45
6	130	85	97	79			87	101	60	47	63	36	55
7	91	76	43				96	89	49	30	50	76	68
8	111	83					87	95	73	33	110	—	55
9	—							80		60	67	—	58
10										77	50	52	72
11										20	41	34	97
12										—	—	—	
Average, *per cent*...	80	82	88	76	83	94	83	88	60	48	73	38	69
		87		90		94	81	95	71	37	90	43	61

— = sample not done.

* Mice.

it is unlikely that there was as much as 0.01 mg. present at one time in the body.[5] The tests did not exclude the possibility that this quantity, or somewhat more, was absorbed and excreted over a longer period than 18 hours, but at such a slow rate as to prevent discovery.

Feces.—The failure to find the specific substance in either the serum

[5] That is, outside the gastrointestinal tract.

In view of the fact that mice could not be immunized by feeding the specific substance of Type I, it was desirable to learn whether destruction of the substance takes place in the gastrointestinal tract of this animal. The method of analysis was the same as for rat feces.

TABLE IV

Resistance to Pneumococcus Type I, Following Ingestion of Soluble Specific Substance Recovered from Feces of Rats to Which It Had Been Previously Fed

Dose	Part 1				Part 2					
	C		E		C		C		E	
	Weight	Result	Weight	Result	Weight	Result	Weight	Result	Weight	Result
cc.	gm.		gm.		gm.		gm.		gm.	
10⁻⁹	82	S			78	S				
	83	S			78*	5				
10⁻⁸	101	2	81	S	81	2	80	2	82	S
	98	2	85	S	88	2	78	2	70	S
10⁻⁷	106	2	93	2	97	S	83	2	90	S
	103	2	91	S	90	2	86	4	87	2
			98	S					85	S
10⁻⁶	108	2	107	S	98	2	89	2	91	S
			104	S			90	2	92	S
			105	S			88	2		
10⁻⁵	111	2	121	2	104	2	94	3	98	2
			133	3			100	2	95	2
							101	2	97	S

Test was done 4 days after the 2nd of 2 feedings	Test was done 3 days after the 2nd of 2 feedings. The C rats in the 1st column received nothing. Each C rat in the 2nd column was fed 4.25 cc. Type I antiserum on each of 2 successive days

Parts 1 and 2 of this table give the data of two experiments done at different times.

* Pneumococcus in heart blood.

Experiment 5.—0.25 mg. of S.S.S. Type I, dissolved in a 1:10 dilution of phosphate solution, pH 7.7, was fed to each of 4 mice, and the same quantity added to the feces of a control mouse. Maximum and minimum values were 95 per cent and 73 per cent, with a final average of 83 per cent (Table II).

Experiment 6.—The experiment was repeated under the same conditions, in which two were used. The maximum and minimum values were 103 per cent and 83 per cent, with a final average of 93 per cent as compared with both control (Table II).

Judging from these data, it seems that the quantities recovered are about the same as in the case of rats, and there is consequently no reason to believe that destruction of the material in the gastro-intestinal tract plays a part in the failure to immunize mice.

Two experiments were done in which the specific substance of Type II pneumococcus was fed to rats.

Experiment 7.—0.5 mg. S.S.S. Type II, to each of 8 rats on each of 2 successive days. On account of the large bulk, the feces were extracted with 100 cc. normal HCl. There were 2 controls. The maximum and minimum values were 96 per cent and 65 per cent, with final average of 85 per cent and 81 per cent (Table II).

Experiment 8.—0.5 mg. S.S.S. Type II, to each of 9 rats on each of 2 successive days. There were 2 controls. Maximum and minimum values were 101 per cent and 77 per cent, with final averages of 88 per cent and 95 per cent.

These figures show that Type II polysaccharide is eliminated in the feces of rats in the same proportion as Type I.

Values for the excretion of Type III polysaccharide were lower than for Types I and II, as the following experiments illustrate.

Experiment 9.—0.43 mg. S.S.S. Type III, to each of 8 rats on each of 2 successive days. The maximum and minimum values were 85 per cent and 43 per cent, with a final averages of 60 per cent and 71 per cent[7] (Table II).

Experiment 10.—0.5 mg. S.S.S. Type III, on 1 day followed by 0.45 mg. the next day to each of 11 rats. The maximum and minimum values were 77 per cent and 20 per cent, with final averages of 48 per cent and 37 per cent (Table II).

Experiment 11.—0.5 mg. S.S.S. Type III, to each of 9 rats on each of 2 successive days. The maximum and minimum values were 110 per cent and 41 per cent, with final averages of 73 per cent and 90 per cent (Table II).

Experiment 12.—0.5 mg. S.S.S. Type III, on each of 2 successive days to each of 8 rats. Maximum and minimum values were 76 per cent and 22 per cent with final averages of 38 per cent and 43 per cent (Table II).

Experiment 13.—0.5 mg. S.S.S. Type III, on each of 2 successive days to each of 11 rats. Maximum and minimum values were 97 per cent and 45 per cent, with final averages of 69 per cent and 61 per cent (Table II).

[7] The resistance of the fed rats in this and succeeding Type III experiments is recorded in Table II of Reference 5.

DISCUSSION

Judging from the results obtained in these experiments, it can be concluded that at no time, up to 6 hours after the ingestion of 1 mg. of soluble specific substance of Types I, II and III pneumococcus, was there present in the serum a quantity sufficient to make a concentration of 1 part in 2 millions. This fact, coupled with the negative results obtained with the urines up to 72 hours after the feedings, makes it probable that none of the material was absorbed from the intestinal tract. This opinion is strengthened by the fact that quantities up to 84 per cent of Type I, 87 per cent of Type II and 59 per cent of Type III specific polysaccharide could be recovered in the feces.[8] In spite of this evidence, however, it is conceivable that quantities too small to be detected were absorbed. The lower value for Type III substance is difficult to explain. An unsuitability of the general method used for this particular type of polysaccharide is not likely to be the cause since this would apply to the control feces as well.

There does not appear to be any direct relation between the quantity of polysaccharide found in the feces and the survival or death of the animal when tested with the virulent organism of any of these three types.

The available data suggest the tentative conclusion that certain cells in the gastrointestinal tract are active in the production of the increased resistance which follows the ingestion of the specific substances of Types I and III.[9] The apparently unchanged state in which this material leaves the intestinal tract, as determined by its activity when fed to new groups of animals, arouses curiosity as to its mode of action.

One of the reasons for making the present investigation was to learn whether it would be contraindicated to undertake the suggested therapeutic measure of feeding a pneumococcus vaccine during the early stages of human pneumonia. If the human being also fails to absorb the polysaccharide of ingested pnemococci, such a procedure loses the principal element of risk which first seems to characterize

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[8] Average of all final averages for Types I (only rats), II and III.

[9] Regarding Type II, see Reference 5.

ii. Healthy persons apparently suffer no ill effects following ingestion of large doses of acid-killed pneumococci [6].

SUMMARY

1. Rats, when fed the soluble specific substance of Types I, II and III pneumococcus, excrete a very large proportion in the feces.

2. Following ingestion of these substances, tests sensitive enough to detect 1 part in 2,000,000 of serum, and 1 part in 3,000,000 of urine, were negative.

3. The polysaccharide of Type I, when recovered from the feces, is as active an immunizing agent as it was originally.

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UREA TOLERANCE AFTER UNILATERAL NEPHRECTOMY IN RABBITS

HOWARD T. KARSNER, M.D., EDITH STRAW,* ROBERT A. MOORE,* M.D., and PAYSON L. HAYSAL, Ph.D.

From the Institute of Pathology, Western Reserve University, Cleveland

It is well established that following the removal of one kidney the remaining fellow increases in size and weight and the glomeruli undergo enlargement (1) without augmentation of their number (2). Usually the remaining kidney exhibits some functional inadequacy for a short time after the operation. The work quoted by and reported by Addis, Myers and Oliver (3) shows that after full recovery the one kidney excretes urea in practically normal amounts. The remaining, usually enlarged, kidney is spoken of as hypertrophic, but the term hypertrophy, if critically employed, implies a connotation of increased functional capacity. The question arises as to whether the remaining kidney exercises only the reserve power of one of paired organs or whether it is capable of an increased functional activity in the presence of demands in excess of those imposed by ordinary body processes. The problem is thus related to the general pathology of hypertrophy rather than to a test for determination of amount of renal tissue. It was decided that the simplest and most precise method of administration of excess of urea is by the intravenous route. For the purposes of this series of experiments it was thought that the curve of urea level in the blood, the urea tolerance curve, drawn as the result of hourly readings over a period of 8 or 9 hours would provide the necessary information.

Method

Rabbits which weighed from 1.7 to 2.5 kilos were employed. After a fast of 48 hours, urea was injected in a dose of 500 mg. per kilo of body weight. 1 hour thereafter and at hourly intervals for 6 to 9 hours, the blood urea was determined

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*Crile Research Fellow in Pathology.

by the aeration method of Myers (4), and simple graphs plotted. In normal animals the blood urea level returns essentially to normal in about 8 hours. Without urea injections, hourly withdrawal of blood over a period of 8 hours shows a maximum variation of only 5 mg.

One group of fourteen animals was subjected to right nephrectomy by the lumbar route under ether anesthesia. Another group of nine animals was subjected to deep incision through the right lumbar muscles under ether anesthesia. The third group of four animals served as controls, in addition to the preliminary
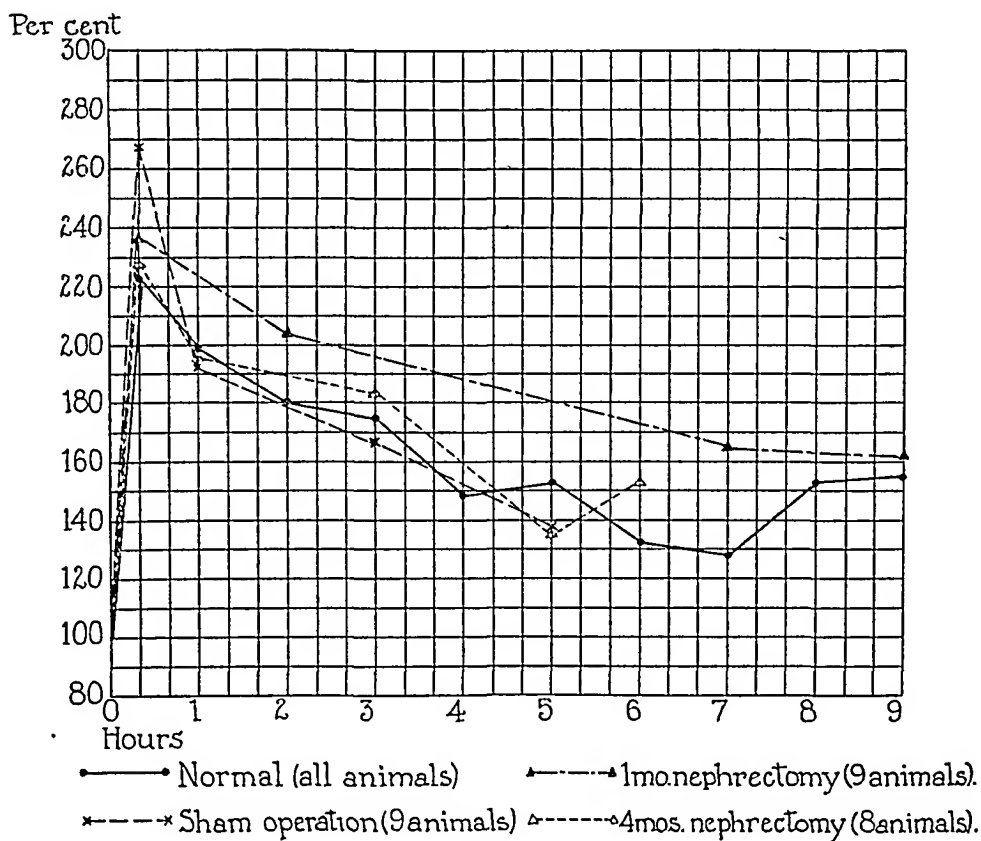
CHART 1

control observations on the other twenty-three animals. The animals were studied before operation and at periods of approximately 1 and 4 months after nephrectomy or sham operation.

The fasting level of urea in the rabbit varies considerably, but in the normal and experimental groups the curve after the urea injections shows respectively the same general course. For comparison, the fasting level is regarded as representing 100 per cent. In the control series 100 per cent represents an average of 43.7 mg. per 100 cc. blood. In the sham operation series it represents 45.5 mg. In the

animals studied 1 month after nephrectomy it represents 63.7 mg., and in those 4 months after nephrectomy 47.4 mg. All values for each animal were based upon at least two curves before operation and one, two or three curves at the specified periods after operation.

RESULTS

The essentials are shown in Chart 1. Immediately following the intravenous injection of 500 mg. urea per kilo of body weight there is in all groups of animals an elevation of blood urea level of from 225 to 267 per cent of the fasting level. In normal animals this falls progressively to 129 per cent in 7 hours with a subsequent elevation. This late elevation is unexplained save as the result of inadequate number of observations, despite the use of twenty-seven normal animals. In the animals subjected to sham operations the initial rise is to a figure higher than in any of the other groups, but the figure falls to within the normal range in 5 hours. For the period of 2 to 4 weeks after unilateral nephrectomy, the curve is decreased, is less sharp, and presumably the rate of decrease slower, than is true of the normal controls and at 7 hours is still significantly higher than the control figures.

SUMMARY

The method of study has an objective somewhat different from, and lacking the precision of, the ratio of Addis and the urea clearance of Van Slyke. It serves, however, to demonstrate that although for a month after unilateral nephrectomy the remaining kidney shows diminished capacity to hold blood urea within the normal range, nevertheless after 4 months this function is maintained in essentially the same degree as if both kidneys were present. This does not imply that all activities of the kidney remaining after unilateral nephrectomy are potentially augmented, but the data offered justify the conclusion that as the remaining kidney undergoes enlargement its functional capacity increases and the process represents a genuine hypertrophy in the critical sense.

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THE CORRELATION BETWEEN THE HISTOLOGICAL CHANGES AND THE FATE OF LIVING TUBERCLE BACILLI IN THE ORGANS OF TUBER- CULOUS RABBITS

By MAX B. LURIE, M.D.

(From The Henry Phipps Institute of the University of Pennsylvania, Philadelphia)

PLATES 1 TO 4

By means of cultural methods it was found in a previous study (1) that multiplication of tubercle bacilli follows intravenous inoculation of rabbits with either the human or the bovine type. At first the human bacillus grows faster than the bovine but soon a change takes place causing its destruction, first in the liver, spleen and bone marrow and later in the lung and kidney. The bovine bacillus multiplies in these organs more slowly; it is destroyed more slowly than the human type in the liver, spleen and bone marrow and it continues to multiply in the lung and kidney without effective opposition until the death of the animal.

Lubarski and Korshinskaja (2) using both similar and other methods have recently confirmed these results as far as they have studied them. Moreover an examination of the protocols of Lewis and Sanderson (3), who came to the conclusion from histological observations that the human tubercle bacillus fails to multiply appreciably in the lungs of rabbits, shows a definite though slight primary increase followed by a decrease in the number of stainable bacilli observed. As will be shown, there is a general parallel between the number of colonies isolated and the number of stainable bacilli, though the culture method, which indicates the viability of the bacilli, is essential to determine quantitative relations.

The study reported here is an attempt to correlate the histopathological changes in the various organs of rabbits with the number of viable bacilli contained in them as indicated by the number of col-

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onies isolated from each organ at various intervals following infection. The end in view is to determine if possible what reactions of the host are associated with the multiplication of the parasite and what with its destruction. The following brief discussion will indicate how terms have been used and what significance has been attached to acid-fast particles.

Cells and Acid-Fast Material Observed

The most significant cell was designated the large mononuclear (Fig. 4). It is twice the size of a red blood cell, with a vesicular, rounded, oval or kidney-shaped nucleus containing sometimes one or several nucleoli, and pale transparent vacuolated cytoplasm with numerous processes.

Sabin and her coworkers (4) have separated this mononuclear phagocyte of the connective tissue into two types, the now well known monocyte and clasmatocyte, which, they have maintained, behave differently toward neutral red with the supravital stain and have different origins and different physiological functions. However numerous investigators, among these Lewis, Willis and Lewis, Carrel and Ebeling, Gardner and Smith, Clark and Clark (5), by the same and by different methods have come to the conclusion that they represent merely physiological states of the same cell. Recently Forkner (6), associated with Sabin, has stated that under certain conditions monocytes may be transformed into clasmatocytes. Injecting neutral red intravenously into rabbits according to the method of Cash and Gardner (7), we have found that at the periphery of advancing tubercles most of the large mononuclears showed the stain, like the monocyte, as fine, even sized granules, arranged spherically, of a uniform salmon pink, but that others showed granules characteristic of the clasmatocyte, being uneven in size, arranged in no definite pattern, and varying in color from red to yellow. In this paper the term mononuclear has been used without the subdivision into monocyte and clasmatocyte.

By gradual transitions the large mononuclear is transformed into what is designated here as the young epithelioid cell (Fig. 4). This is a much larger cell with abundant foamy or reticulated cytoplasm. The nucleus is usually round and very poor in chromatin. These cells are connected with one another by large processes. They are seen in the early tubercles. Later they become rounded and, as first described by Castrén (8), the cytoplasm becomes differentiated into an inner more eosinophilic, more compact, spherical area and a peripheral zone of more basophilic, often vacuolated cytoplasm. It is in the central area that the large accumulation of very finely divided neutral red granules is seen when the cells are stained supravitally, as shown by Lewis, Willis and Lewis (5) and by Sabin and her coworkers (4). This is the mature epithelioid cell (Fig. 5).

One other cell plays a part in the tuberculous process in the lung. This is the alveolar phagocyte, a large spherical cell with foamy, abundant, pale staining

cytoplasm, and an eccentric oval or kidney-shaped vesicular nucleus. Carbon particles and other ingested material are often found in the cytoplasm. Believing with Lang (9) and with Gardner and Smith (5) that this phagocyte is derived from the so called septal cell belonging to the general group of connective tissue phagocytes or clasmatocytes, we have designated it simply as a macrophage.

In these studies various forms of acid-fast material in addition to typical acid-fast rods were noted within the cells. These ranged from ovoid or globular, deeply or lightly staining forms of the size of large cocci, or larger, to irregular granular debris with all the outward characteristics of fragments of acid-fast bacilli, and to fine dust-like, scarcely visible, acid-fast particles. They showed a tendency to change in the order given.

As to the significance of this material, little can be said with certainty at the present time. There are numerous observations in the literature suggesting that such particles are degeneration products of the tubercle bacillus. Amongst these are the experiments on "lysis" (10), showing that when tubercle bacilli are introduced into the tissues or cavities of tuberculous animals there is a rapid accumulation of acid-fast material and a marked reduction in the numbers of stainable bacilli associated with the heightened resistance of the infected animal to reinfection. In normal animals, similarly treated, intact bacilli persist and granular debris is much less in evidence but acid-fast material interpreted as degeneration products of the tubercle bacillus has been observed in the cells by numerous investigators (11, 12). An objection that has been urged to this interpretation even with the reinfected resistant animal, is that when inoculated into guinea pigs this material gives rise to progressive tuberculosis. It has been shown by the writer (13) however, that the tubercle bacilli of reinfection are in great part destroyed immediately, and that some few bacilli linger in the tissues. These would account for the virulence of such particles. On the other hand Spengler's "splitters" (14), the work of Sweany (15) and especially the recent single cell studies of Kahn (16) would suggest that they may be stages in the development cycle of the microorganism.

In the present studies the occurrence of acid-fast material is correlated with the number of living bacilli cultured. In general it has been found that the bacillus grows more slowly and is destroyed more rapidly in that organ in which more acid-fast material accumulates within a given time.

Method

Three strains of tubercle bacilli were used, a modified strain of the bacillus of Calmette and Guérin (BCG), one of human type, P-48A, and one of bovine type, Bovine C. The last two have been described in a previous paper (13). The BCG was obtained from Dr. W. H. Park in March, 1926. At that time it caused no progressive tuberculosis in guinea pigs, but instead of its being propagated under the conditions prescribed by Calmette, it was transplanted monthly on glycerol agar, and when the animals were infected in the summer of 1927 it had attained

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the nodules are composed of a thick, closely packed ring of mononuclears, many in mitosis, surrounding young epithelioid cells; these are the early tubercles (Fig. 4). Tubercle bacilli are found within the young epithelioid cells in large numbers, and in smaller numbers within the mononuclears. The simultaneous occurrence of acid-fast rods and acid-fast granular debris in the same cell suggests that some bacilli are being destroyed.

The microorganisms have been greatly reduced in number (2,930 colonies) by the end of the 2nd week (Fig. 2). The epithelioid cells have assumed a more mature character, and the ring of mononuclears is very thin or has disappeared entirely. The first signs of caseation appear (Fig. 6): there is an accumulation of polymorphonuclears in some tubercles. Isolated tubercle bacilli may be seen in some of the mature epithelioid cells, especially in those undergoing pyknosis or necrosis, or they may be absent altogether. Exudation into the alveoli has become conspicuous.

The most extensive tuberculous changes are found in the lung 4 and 6 weeks after infection, when most of the bacilli have been destroyed (Fig. 3). At 4 weeks (655 colonies) the parenchyma is largely replaced by tubercles with or without central foci of caseation. Langhans' giant cells appear at the periphery. The tubercles are invested by lymphocytes, often permeating the epithelioid cells, and sometimes ingrowing capillaries, plasma cells and fibroblasts are seen. Mitosis is rare. The exudate into the alveoli is more widespread. The alveolar lining in contact with tubercles may show metaplasia with cuboidal and columnar cells. Tubercle bacilli are very difficult to find in the epithelioid cells.

At the 6th week (1,000 colonies) many tubercles are composed of widely spaced, disintegrating epithelioid cells, and they are often permeated by lymphocytes and polymorphonuclears, and canalized by capillaries in all directions. Giant cells are more numerous at the periphery and sometimes occur in the centre about the remains of caseous material (cf. Medlar (26)). The exudation at times assumes pneumonic proportions, and may undergo caseation. Tubercle bacilli are found in small numbers in epithelioid cells in contact with caseous foci of interstitial tubercles and at the edge of pneumonic areas undergoing caseation.

In one rabbit 2 months after infection only fourteen colonies were recovered after sodium hydroxide treatment of the tissue. The lung field has cleared considerably. The disintegrating epithelioid cells sometimes contain minute, faintly acid-fast globules staining dark brown with hematoxylin-eosin, often clumped in hollow spheres and having no resemblance to derivatives of tubercle bacilli. Often the entire tubercle is composed of such cells surrounded by a thick ring of lymphocytes. Tubercle bacilli in small numbers are seen in immediate contact with caseous areas. Caseous foci may disintegrate into microscopic cavities (Fig. 7).

The data show that although tubercle bacilli are being destroyed from the very beginning, proliferation continues to outstrip destruction until after 2 wee__ __d cells are formed.

In any two rabbits, even at the same interval, the more effective destruction of bacilli has occurred in that animal in which epithelioid cell and tubercle formation are further advanced. The most rapid growth of the microorganism was found in those tissues in which the most extensive formation of new mononuclears was observed. When the parasite stops multiplying, mitosis is seen less often and now giant cells appear at the periphery of the tubercle. Caseation and exudation into the alveoli are coincident, not with the greatest increase, but with the most rapid destruction of bacilli. Caseation is therefore not due to the mere accumulation of tubercle bacilli, though some are usually present in or about a caseous focus. Epithelioid cells persist for a considerable time after most of the bacilli within them have died, although their disintegration products may still be present. Lymphocytic infiltration and granulation tissue are seen after the greater part of the tubercle bacilli have been destroyed.

The Lung after Infection with Tubercle Bacilli of Human Type

With a dose of 0.01 mg. of human tubercle bacilli there is little multiplication of the microorganism during the 1st week. The associated slight histological changes are of the same character as those described for the BCG.

No gross tuberculosis is seen at the end of the 2nd week. The bacilli have increased ten and thirty times. In Rabbit 25–76, from which the larger number of colonies (1,580) were isolated, the histological changes are essentially those seen in the 1st week with the BCG except that cells lying free in the alveoli contribute considerably toward the tubercle formation. Tubercle bacilli are again found in large numbers in the young epithelioid cells, three or four in a cell, together with acid-fast particles. Macrophages in the alveolar space occasionally contain a bacillus. The lymph follicles are much enlarged. In them large mononuclear cells show frequent mitosis, and there are islands of young epithelioid cells, some containing numerous tubercle bacilli as well as carbon particles. There is no caseation.

In Rabbit 23–01, with 560 colonies at this interval, the infiltration into the alveolar septa is less, mitosis is rarer, the tubercles are fewer and composed more largely of epithelioid cells, in which tubercle bacilli are seen in smaller numbers than in the first rabbit.

In one rabbit 4 weeks after infection the bacilli had either remained stationary in number or had decreased considerably; in the other, they had increased to 7 and 22 times the numbers found in the 2nd week.

In the second rabbit (No. 25–79, 12,500 colonies) the tuberculous process is of two types: interstitial or productive, and intraalveolar or pneumonic. The for-

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croscopically the productive and pneumonic processes are more extensive and further advanced. Tubercle bacilli are difficult to find in the mature epithelioid cells of the interstitial tubercles; caseation when present is usually slight; the tubercle is surrounded by mononuclears with occasional masses of lymphocytes within vessels and free in the tissues. The greater part of the lesion involves both interstitial tissue and alveolar lumina. In the alveoli, macrophages contain a great deal of acid-fast globules, but the collections of epithelioid and giant cells show little if any. Tubercle bacilli are not found within these epithelioid cells, except occasionally, in moderate numbers, in tubercles undergoing caseation.

Thus with a far more extensive epithelioid cell and tubercle formation than in the 2nd week, viable bacilli have not increased and persist appreciably only in the alveolar pneumonic areas.

In one rabbit 6 weeks after infection the bacilli had increased to twelve times, in the other to only twice the number found after 4 weeks. In the first (No. 22–50, 12,000 colonies) the process is essentially as in the previous interval, but with caseous pneumonia predominating. Tubercle bacilli are found even in the interstitial tubercles, and not only in the epithelioid cells in the zone between the caseated and the intact cells, but also in the unaffected epithelioid cells in an area of slight extent, most of the tubercle having caseated. These tubercles are invested by a growing zone of mononuclears infiltrated with polymorphonuclears; giant cells are not found at the periphery; lymphocytic investment is only partial. Tubercle bacilli are numerous in the pneumonic areas, especially those undergoing caseation. There may be metaplasia of the alveolar lining in areas of tuberculous pneumonia and the bronchi may contain cells similar to those in the alveoli carrying tubercle bacilli.

In general there was extensive caseation, few intact epithelioid cells and a massive growth of mononuclears. The epithelioid cells have apparently failed to check effectively the growth of the microorganism, especially in the alveoli, where tubercle bacilli are numerous within epithelioid cells and mononuclears. The progress of the lesion has not been stopped.

In the second rabbit of this interval (No. 22–85) only 2,100 colonies were isolated. The process is largely interstitial with the pneumonic process and caseation much less extensive than in No. 22–50. Tubercle bacilli are found much less frequently, even in the pneumonic areas.

Essentially the same difference is found between the lungs of rabbits killed 2 months after infection, from one of which 200,000 colonies were isolated and from the other 3,100. The first (Fig. 11) showed a massive caseous pneumonia with much less extensive interstitial change. The productive process consists of multiple foci of caseation separated from one another by tuberculous granulation tissue, the advancing edge of this tissue being a mass of mononuclears with frequent mitosis and many polymorphonuclears. Tubercle bacilli are found in great numbers in the intact epithelioid and giant cells that have collected in the alveoli in the areas of caseous pneumonia. In the pneumonic areas the exudate is sometimes largely serum and polymorphonuclears intermixed with macrophages.

In the second rabbit, in which the growth of bacilli was held in check, the lung, though as massively consolidated as in the first rabbit, was the seat of an interstitial process (Fig. 12) with the tuberculous pneumonia usually limited to areas immediately about the tubercles, and even these few foci of caseous pneumonia had become the centers of a peripheral productive change.

As here shown the same defensive processes that have been observed in the lung with the BCG and human strains are overcome characteristically with the bovine. The initial reaction of the organ to the most virulent strain is more intense as expressed by a greater rush of polymorphonuclears and a more extensive multiplication of mononuclears. But also from the very beginning cellular accumulation within the alveoli, in which, as was seen before, tubercle bacilli are not readily destroyed, is more important in the bovine-type lesion, which is more diffuse and less nodular than with the human strain. These changes were accompanied by a sharp increase of viable tubercle bacilli in the 2nd week following a lag in the 1st week. They stopped multiplying between the 2nd and 4th week, again at a time of extensive formation of epithelioid cells and of tubercles, but they persisted in the epithelioid cells collected in the alveoli. From now on the lesion is increasingly pneumonic and bacilli are found in increasingly large numbers. Again they are fewer in the interstitial tubercles but even in these more numerous when the pneumonic areas are more extensive. The greater part of the parenchyma may become consolidated by caseous pneumonia, throughout which tubercle bacilli are found in tremendous numbers. On the other hand, they have been held in check in those rabbits in which the interstitial lesion predominates, even though the parenchyma may be as extensively involved.

The difference between the human and bovine types is essentially one of degree. Usually with the former the process is chiefly interstitial; it is circumscribed and never becomes generalized. The pneumonic areas when present are localized and tend to become surrounded by a productive process. Caseation is limited; the regeneration of mononuclears soon ceases; giant cells appear at the periphery; lymphocytes and granulation tissue permeate the tubercle; in some instances after the lesion has persisted 2 months, softening and cavity formation overtake the isolated residual tuberculous foci. With the bovine infection, the lesion is chiefly of a mixed pneumonic and productive

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character; it spreads over almost the entire lung; caseation appears early and becomes massive. The zone of intact epithelioid cells is slight and regeneration of mononuclears goes on unabated. Giant cells do not form at the periphery of the interstitial tubercles, though they have been seen in the pneumonic lesion. Lymphocytes do not permeate the tubercle to any considerable extent and the granulation tissue appears as islands between the multiple foci of caseation. The caseous pneumonia is widespread throughout the lung. Softening and excavation were not noted within the course of the experiment. Associated with these differences is the effective destruction of the human bacillus within the interstitial lesions. With the bovine on the other hand the bacilli are held in check but not destroyed in these lesions, whereas in the pneumonic areas they accumulate in far greater numbers than the bacilli of human type.

The Host-Parasite Relationship in Other Organs after Infection with the Three Strains

Essentially the same correlations between histological changes and the fate of tubercle bacilli as found in the lungs were seen in the other organs involved, and they are summarized here. When not otherwise specified the processes are like those in the lung and the reaction of each organ to the three strains differs only in degree. Significant differences are briefly described.

Liver.—The initial inflammation is very much less than in the lung despite the localization of larger numbers of bacilli in the liver. From the beginning bacilli are destroyed more effectively as shown by the much larger accumulation of acid-fast material in the Kupffer cells than in the pulmonary mononuclears. Associated with the slower multiplication of bacilli is the formation of fewer mononuclears and of smaller though more numerous tubercles, and the early appearance of giant cells (in the 1st weeks with the BCG and from the 2nd to 4th week with the human and bovine). The whole tubercle is often composed of giant cells; isolated giant cells are also seen. Caseation is not found with the BCG, presumably because little remains of the bacillus after the onset of extensive destruction. With the human infection, associated with the somewhat larger numbers of viable bacilli recovered, caseation and central softening with the formation of tissue defects are seen after 4 weeks, simultaneously with the greatest destruction of bacilli. The bovine infection differs from the human in the greater accumulation of acid-fast particles in the Kupffer and epithelioid cells and in the larger number and size of tubercles and their longer persistence, in association with the larger numbers and

longer persistence of viable bovine bacilli. With all three strains the bacilli are almost completely destroyed, and the liver tubercle, unlike the pulmonary, completely disappears between the 6th and 8th week.

Spleen.—In the spleen the initial growth of tubercle bacilli is more rapid than in the liver but slower than in the lung, and bacilli persist longer than in the liver but are more quickly destroyed than in the lung. This is explained, in part at least, by the different reactions of the splenic pulp and corpuscle. The macrophages of the pulp at first very conspicuously contain round acid-fast globules as big as large cocci, which later break up into irregular round acid-fast globules as in the epithelioid and giant cells as fine ductlike, scarcely visible debris. These particles are frequently found together with intact tubercle bacilli in the same cell. With a presumably greater localization of bacilli in the pulp corresponding with that of particulate matter injected into the venous system (19), the tubercles are at first more numerous than in the corpuscle. But the pulp tubercles mature earlier, show less active formation of mononuclears, are soon transformed into giant cells (Fig. 17) and tubercle bacilli are more rapidly destroyed within them. The tubercles remain small and stain darker than those in the corpuscle, the cells often containing acid-fast particles, red blood cells, hemoglobin pigment and other ingested material. Living bacilli persist longer and there is less acid-fast material in the tubercles of the corpuscle, which become massive, involving the whole structure.

With the BCG, slight caseation appears in the corpuscle tubercles 4 to 6 weeks after infection. With the less complete destruction of bacilli of human type, caseation is more extensive in the corpuscle and central softening appears 4 weeks after infection. With the bovine infection also tubercle bacilli disappear from the epithelioid cells in the corpuscle in some rabbits. In rabbits even of the same interval the destruction of bacilli is more complete in that animal in which, as indicated by the abundance of epithelioid cells, tubercle formation is further advanced (Figs. 15 and 16). In other rabbits the corpuscle tubercles, as well as tubercles in other organs, failed to destroy the microorganism and isolated tuberculous foci containing large numbers of living bacilli persisted even 2 months after infection. Caseation was not necessarily found with large numbers of bovine bacilli but often in association with a more mature tuberculous process and fewer bacilli. Central softening with the formation of tissue defects was noted in the splenic corpuscle 4 weeks after infection in tubercles permeated by lymphocytes, polymorphonuclears and capillaries, where numerous living tubercle bacilli persisted within the epithelioid cells.

Bone-Marrow.—With the BCG and human strains there is a considerable accumulation of acid-fast material in the reticular cells 1 week after infection. Growth and destruction of bacilli were seen with the same histological changes as in the other organs. No caseation was noted, again apparently because too few bacilli remained after the onset of extensive destruction.

With the bovine infection acid-fast granules are at first very prominent in the

reticular cells. From the 4th week on the bacilli are effectively destroyed in the epithelioid cells in some rabbits. In these caseation is seen synchronously with the greatest destruction of bacilli. Later these tubercles completely disappear together with the bacilli. In other rabbits the bovine bacilli are not destroyed, mitosis continues and, as in the lung with human infection, large numbers of living bacilli persist even 2 months after infection within the epithelioid cells of tubercles extensively permeated by lymphocytes and granulation tissue. There is little caseation in these tubercles and it is found not where intact bacilli are most numerous but where there are most acid-fast particles with all the characteristics of disintegrating bacilli.

Kidney.—The host-parasite relationship in the kidney is modified by two factors: the initial minimal localization of bacilli, and the anatomy and physiology of the organ. The first determines a long lag period in the progress of the disease. With maturation of the tubercle the bacilli are held in check for 4 weeks. However, due to the intertubular origin and extension of the tubercle, many tubules and glomeruli become involved. With the disintegration of foci of caseation in the cortex, tuberculous material may break into these channels and may be carried by the flow of urine to the medulla, where it sets up a new process (Fig. 19). Furthermore casts form within the involved tubules, in which tubercle bacilli may multiply as in a culture medium. Thus bacilli may persist in this organ for a long time, even when the cortical tubercles are healed. Central softening develops in the kidney except with the bovine infection.

With the human type the first stages of caseation are synchronous with the greatest accumulation of bacilli. However since this was not found until the 4th week, when caseation had occurred throughout the body, this observation is not evidence for an initial toxicity of the bacillus to the tissues. There was no constant relationship between the numbers of bacilli in the lung and in the kidney. (In 2 months, 5,260 colonies were cultured from the lung and none from the kidney, which contained only one minute cortical tubercle.) There was no evidence that these lesions in the kidney are caused by bacilli brought from the lungs or other organs to the kidney.

The bovine bacilli tend to increase from the 4th week, and they regularly persist within the epithelioid cells. Yet in some rabbits they may be largely destroyed by the epithelioid cells. Caseation occurred in the kidney 4 weeks after inoculation in the presence of relatively few bacilli (297 colonies).

SUMMARY AND DISCUSSION

It has been found that although there is some parallelism between the quantity of tubercle bacilli demonstrable histologically and the number of colonies that can be isolated from a given tissue, the culture method is far the more efficient in indicating quantitative relations. Tubercle bacilli were not perceived in the organs of rabbits

1 day after infection with the modified BCG although as many as 1,500 colonies were isolated from one of them. This may be solely because it is difficult to see widely dispersed single minute acid-fast rods in the diffuse infiltrations of mononuclears with their hyperchromatic nuclei and sparse cytoplasm. Later, with the formation of tubercle, the parallelism is much closer. The culture method gives evidence concerning the number of living tubercle bacilli in the tissue.

The significance of the accumulation of acid-fast particles in the tissues has been discussed. It has been seen that from the beginning this accumulation is greater in the Kupffer cells of the liver, in the macrophages of the spleen and in the reticular cells of the bone marrow than within the mononuclears of the lung, the organ where the bacilli grow with the greatest rapidity and are destroyed with the greatest difficulty. Acid-fast particles are more prominent with the bovine than with the human bacillus or the BCG, the microorganism that is destroyed with the greatest difficulty thus leaving more incompletely digested bacillary debris at a given time within the cells. Thus it seems permissible to conclude from the presence of acid-fast material that some tubercle bacilli are undergoing destruction even 24 hours after infection. The initial accumulation of polynuclear leucocytes corresponds with the subsequent severity of the infection. Despite the greater primary localization of bacilli in the liver, this initial inflammatory reaction with all three infections is much greater in the lung than in the liver. In each organ it is more intense with the bovine than with the less virulent strains.

The multiplication of the bacillus and its accumulation within large mononuclear and young epithelioid cells is accompanied by an intense formation of new mononuclears by mitosis. The more rapid the growth of the bacillus, the more conspicuous the regeneration of these cells. Thus with all strains mitosis is more intense in the more susceptible organ, as in the lung compared with the liver; with the most virulent strain the most extensive and diffuse accumulation of these new cells corresponds with the greater rise in the numbers of bovine bacilli after the lag of the 1st week.

With the maturation of the epithelioid cells and the formation of tubercles the bacilli have already been greatly reduced numerically

and the speed of this process diminishes with the virulence of the three strains used. The faster the development of tubercle the faster the destruction of the bacillus and the earlier the resorption of the tubercle.

Tubercle bacilli never accumulate in such large numbers in the mononuclears of the liver as they do in the lung. Though at first the tubercles in the liver may be more numerous than those in the lung they never attain the same size. The formation of new mononuclears by mitosis is restricted and Langhans' giant cells appear very early (1st and 2nd weeks). In the lung, giant cells are not found until much later with the BCG and the human bacillus (4th week); they were not noted in the interstitial tubercles with the bovine type, but the extension of these tubercles was accompanied by an unabated mitosis of mononuclears until the death of the animal. The liver tubercles are resorbed early even with the bovine infection. Associated with these histological differences are the slow initial growth and the early and complete destruction of the tubercle bacilli even of bovine type in the liver, and the more rapid initial growth in the lung, with the later destruction of the BCG and the human bacillus and the unabated growth of the bovine bacillus. Similar differences were observed between the splenic pulp and corpuscle. In the former the accumulation of acid-fast particles was much greater and the tubercles developed earlier. Mitosis of mononuclears was less frequent and giant cells appeared earlier. Tubercle bacilli, always intracellular, disappeared from the tubercles in the pulp sooner than from those in the corpuscle, and the tubercles themselves first disappeared from the pulp. Consequently with the persistence of bacilli mitosis continued in the tubercles of the corpuscle and these attained a much larger size.

Moreover individual resistance is linked with the ability to form mature tubercles early. In two animals simultaneously infected with the same strain and killed at the same time, the destruction or retardation of the bacillus is greater in that rabbit in which maturation of the tubercle and of epithelioid cells has proceeded further (Figs. 15 and 16).

These observations indicate that the mononuclears of different organs or even of the same organ, as in the different parts of the spleen, have a different capacity to destroy the tubercle bacillus, and that the

transformation of the mononuclear into the mature epithelioid cell follows its destruction of the tubercle bacilli.

In the lung the more virulent types of bacillus are destroyed within the epithelioid cells of interstitial tubercles but persist in foci of tuberculous pneumonia. In this organ in rabbits infected with the human strain and to a lesser degree in rabbits infected with the bovine strain, the parasite largely disappears from the epithelioid cells of interstitial tubercles. But with both strains tubercle bacilli in large numbers may accumulate within epithelioid cells lying free in the alveoli. With the human type they are numerous within the cells and free in caseous material in the localized foci of caseous pneumonia. With the bovine infection, this caseous pneumonia is more often widespread and in the areas of caseous pneumonia the greater part of the vast accumulation of bovine bacilli in the lungs is found; as many as 200,000 colonies have been isolated from 10 mg. of tissue (Fig. 11). Flooding of the respiratory passages by the caseation of tuberculous lesions into the bronchi plays an important rôle in dissemination of tubercle bacilli through the lung. The process on the contrary is predominantly interstitial when the bovine bacillus is held in check (Fig. 12).

Thus there is apparently some factor acting in the alveoli that favors the growth of the parasite. The accumulation of tubercle bacilli is seen especially in the peripheral epithelioid cells in immediate contact with the alveolar space. In the same lung the bacilli are much fewer in the interstitial tubercles.

The accumulation in human tuberculosis of large numbers of tubercle bacilli in the tissues lining cavities is well known. Novy and Soule (20) have shown that within certain limits the growth of the bacillus *in vitro* is proportional to the oxygen tension of its environment. Corper, Lurie and Uyei (21) have confirmed these observations and have noted further that a difference in the gaseous environment of the bacilli equal to the difference between the conditions existing in the alveolar air and the venous blood is sufficient to cause a considerable increase in the growth of the microorganism *in vitro*. Loebel, Shorr and Richardson (22) by the use of Warburg's manometer have found that the oxygen consumption of tuberculous tissue is such that a tubercle 0.5 mm. thick would completely exhaust the oxygen of the air before it reached the center.

These observations suggest that a factor responsible for the greater multiplication of the bacillus in the cells of the alveoli may be the greater oxygen tension of the alveolar air.

In the liver, spleen and bone marrow even with the bovine infection many instances were found of the effective destruction of the parasite synchronously with the maturation of epithelioid cells and the formation of tubercle. On the other hand, in the spleen and bone marrow of some rabbits, living bacilli persisted within the epithelioid cells of isolated tubercles even 2 months after infection, a condition never found with the human type or BCG infection. Thus the epithelioid cell is the means of defense for the rabbit against the bovine type bacillus, and as such it is usually adequate in the liver, spleen and bone marrow though ineffective in the lung and kidney. In the latter, descending infection, and the occasional colony-like multiplication of bacilli in unorganized material, tubular casts, determine the long persistence of large numbers of bacilli in this organ.

In differentiating the mononuclear phagocyte of the connective tissues into the monocyte and clasmatocyte Sabin and her coworkers (23) have maintained that the clasmatocyte can efficiently destroy the tubercle bacillus but that the monocyte and its derivatives, the epithelioid and Langhans' giant cells, cannot. With the progress of the disease they have noted that the monocytes accumulate in great numbers in the foci of infection and overflow into general circulation (4). White (24) and Sabin and her coworkers have concluded that tuberculosis is specifically a disease of the monocyte, and that this cell and its derivatives act as incubators for the tubercle bacillus. Doan and Sabin (25) have therefore sought, with indecisive results, to protect the body against tuberculosis by an antimonocytic serum. However it has been shown here that although an intense multiplication of mononuclears is associated with the growth of the tubercle bacillus, their transformation into mature epithelioid cells is constantly associated with its destruction, and the rapidity of the destruction varies with the rapidity of the maturation of tubercle. Even in the bovine infection the epithelioid cells destroy the bacilli in the liver, spleen and bone marrow as a rule, and even in the lung, keep them in check in the interstitial tubercles.

The appearance of giant cells is associated with cessation or diminu-

tion of mononuclear regeneration by mitosis, and is coincident with cessation of multiplication or marked reduction in the number of living bacilli. They therefore appear earlier and in larger numbers in these organs or parts of organs that first destroy the bacillus (Figs. 16 and 17). They were not observed even 2 months after the bovine infection in the interstitial tubercles in the lung. Their absence and the continued mitosis of mononuclears, which accounts for the massive pneumonic and interstitial consolidation of the lung with this infection, were associated with the failure of the lung to destroy effectively the bovine parasite. The formation of giant cells in the pneumonic foci in the bovine infection would seem to be an exception to this rule.

The Langhans giant cells have often been considered an indication of the chronicity of the pathological process. It would appear that they are formed from existing epithelioid cells when the multiplication of the bacillus has ceased and the stimulus for the formation of new cells has decreased or stopped. Giant cells were most conspicuous in the liver and splenic pulp where, with the BCG infection, no caseation ever developed, and in the liver before caseation was seen anywhere in the body. In the human and bovine infections, giant cells formed in the liver before caseation appeared. Hence caseation is not a necessary requirement for giant cell formation, as maintained by Medlar (26), though these cells frequently form about caseous material.

Lymphocytes and granulation tissue do not cause the destruction of tubercle bacilli, these being destroyed in their absence. They usually appear about tubercles due to all strains and in all organs, after the greater part of the microorganisms have been destroyed (Fig. 18). The bacilli are not destroyed in the lung with bovine infection where the tubercles are usually little permeated by lymphocytes and granulation tissue. There is however, no constant relation between granulation tissue and destruction of tubercle bacilli, for in the lung after the human infection and even in other organs after the bovine infection isolated tubercles may be surrounded and penetrated by lymphocytes and granulation tissue at a time when considerable numbers of living bacilli are still histologically demonstrable within the epithelioid cells.

Caseation is usually not caused by the local accumulation of tubercle bacilli. At first, when the BCG (after 1 week) and the human microorganism (after 2 weeks) are present in the cells in very large numbers as demonstrated both histologically and by culture (Figs. 4 and 13) there is no necrosis of these cells. An exception to this rule found in the lung with the bovine infection is considered below. Later, after the bacilli have been destroyed to a great extent and even though the number of bacilli is small, caseation appears (Fig. 14). After this preliminary destruction the extent of caseation apparently varies with the number of residual bacilli. With the least virulent microorganism, the BCG, few bacilli remained in the liver in the 4th week and no caseation was seen. In the tubercles of the splenic corpuscle at the same time bacilli were somewhat more numerous and there was scant caseation. On the other hand with the human bacillus after 4 weeks more bacilli survived and caseation was more extensive in both organs; with the bovine microorganism tubercle bacilli were much more numerous and caseation was far advanced.

In the lung, however, caseation appeared with the first considerable accumulation of the bovine bacilli present 2 weeks after inoculation. That the bovine bacillus is primarily more injurious to the lung of rabbits than the BCG or the human bacillus is suggested by the greater intensity of the initial inflammation and by the more conspicuous accumulation of cells in the alveoli evident from the very beginning of infection. Maximow (27) showed that bovine bacilli even in small numbers cause the death of cells in tissue cultures of rabbit lymph nodes whereas the BCG or the human bacillus may accumulate within the cells in tremendous numbers without injuring them. Nevertheless in the liver, spleen and bone marrow of the living animal, caseation does not appear at the time when bovine bacilli are most abundant, but after they have been greatly reduced in numbers.

Large numbers of the less virulent types of tubercle bacilli accumulated in different organs a short time after infection do not cause caseation, and with the bovine infection caseation under the same conditions occurs only in the lung. Later when the animal is sensitized caseation occurs in various organs in the presence of the small numbers of tubercle bacilli that remain in the tissues after most of

them have been destroyed, and the extent of this caseation varies with the numbers of residual bacilli. These observations suggest that a large number of bacilli fail to cause necrosis soon after infection whereas a few bacilli produce caseation in the animal that is sensitized.

Many investigators have held that caseation is due to sensitization. Krause (28), Huebschman (29) and Pagel (30) think that caseation is caused by the action of tuberculin-like substances on the sensitized tissues of the allergic animal. Rich and McCordock (31) view the process in essentially the same light. Recently Schleussing (32) has suggested that caseation is a coagulation necrosis in Weigert's sense of an allergically inflamed tissue, and is similar to the necrosis of the Arthus phenomenon.

CONCLUSIONS

1. The mononuclears of the liver, splenic pulp and bone marrow destroy tubercle bacilli more readily than those of the lung, kidney or splenic corpuscle.

2. The multiplication of tubercle bacilli in an organ and their accumulation within mononuclears is accompanied by active new formation of these cells by mitosis.

3. When these mononuclears are transformed into mature epithelioid cells and tubercles have reached their maximum development the bacilli have already undergone extensive destruction and are disappearing.

4. Tubercle bacilli of moderate virulence (human type and BCG) are usually effectively destroyed within epithelioid cells of all organs. In the lung and kidney bovine bacilli persist within epithelioid cells but in other organs they are usually destroyed.

5. Tubercle bacilli are less effectively destroyed within epithelioid cells collected in the alveoli of the lung than in those forming tubercles in the interstitial tissues.

6. After multiplication of tubercle bacilli has ceased regeneration of mononuclears by mitosis becomes less active and now Langhans' giant cells may be formed from preexisting epithelioid cells.

7. Lymphocytes and encapsulation of tubercles by granulation tissue do not cause the destruction of tubercle bacilli.

8. Immediately after infection accumulation of the less virulent

the tubercle. The epithelioid cells are disintegrating and some contain faintly acid-fast globules clumped in the form of hollow spheres. Magnification about ×200.

FIG. 8. Two interstitial epithelioid cell tubercles in the lung of Rabbit 25–79, 4 weeks after infection with tubercle bacilli of the human type; 12,500 colonies were isolated from this lung. No bacilli could be found in these tubercles. Magnification about ×200.

FIG. 9. An area of caseous pneumonia in another part of the lung shown in Fig. 8. Magnification about ×200.

FIG. 10. Part of Fig. 9 under higher magnification, showing numerous tubercle bacilli in a plug of caseous material within an alveolus. Magnification about ×900.

PLATE 3

FIG. 11. The lung of Rabbit 22–17, 2 months after infection with tubercle bacilli of the bovine type; 200,000 colonies were isolated. Massive tuberculous pneumonia with very extensive caseation of interstitial tubercles. Magnification about ×200.

FIG. 12. The lung of Rabbit 20–95, 2 months after infection with tubercle bacilli of bovine type; 3,100 colonies were isolated. The interstitial process predominates. Caseation is less extensive, fibroblasts and lymphocytes surround the tubercle. Magnification about ×200.

FIG. 13. Malpighian corpuscle of the spleen of Rabbit 25–76, 2 weeks after infection with tubercle bacilli of human type; 1,380 colonies were isolated. Mitosis is frequent; there are no epithelioid cells. Magnification about ×900.

FIG. 14. Spleen of Rabbit 25–79, 4 weeks after infection with tubercle bacilli of the human type; 416 colonies were isolated. Extensive tuberculosis of Malpighian corpuscle with caseation. Magnification about ×140.

PLATE 4

FIG. 15. Malpighian corpuscle of speen of Rabbit 22–75, 2 weeks after infection with tubercle bacilli of the bovine type; 2,410 colonies were isolated. There are no epithelioid cells. Magnification about ×200.

FIG. 16. Malpighian corpuscle of spleen of Rabbit 25–86, 2 weeks after infection with tubercle bacilli of bovine type; 1,425 colonies were isolated. Note extensive formation of epithelioid cells. Magnification about ×200.

FIG. 17. Mature epithelioid and giant cell tubercle in the pulp of the spleen adjacent to the Malpighian corpuscle shown in Fig. 16. Acid-fast particles were present within the cells. Magnification about ×200.

FIG. 18. Liver of Rabbit 22–50, 6 weeks after infection with tubercle bacilli of the bovine type; 2 colonies were isolated. Lymphocytes and giant cells are conspicuous. Magnification about ×200.

FIG. 19. The cortex of the kidney of Rabbit 22–76, 4 weeks after infection with tubercle bacilli of the bovine type; 297 colonies were isolated. The process of descending infection by way of the tubules is shown. Magnification about ×200.
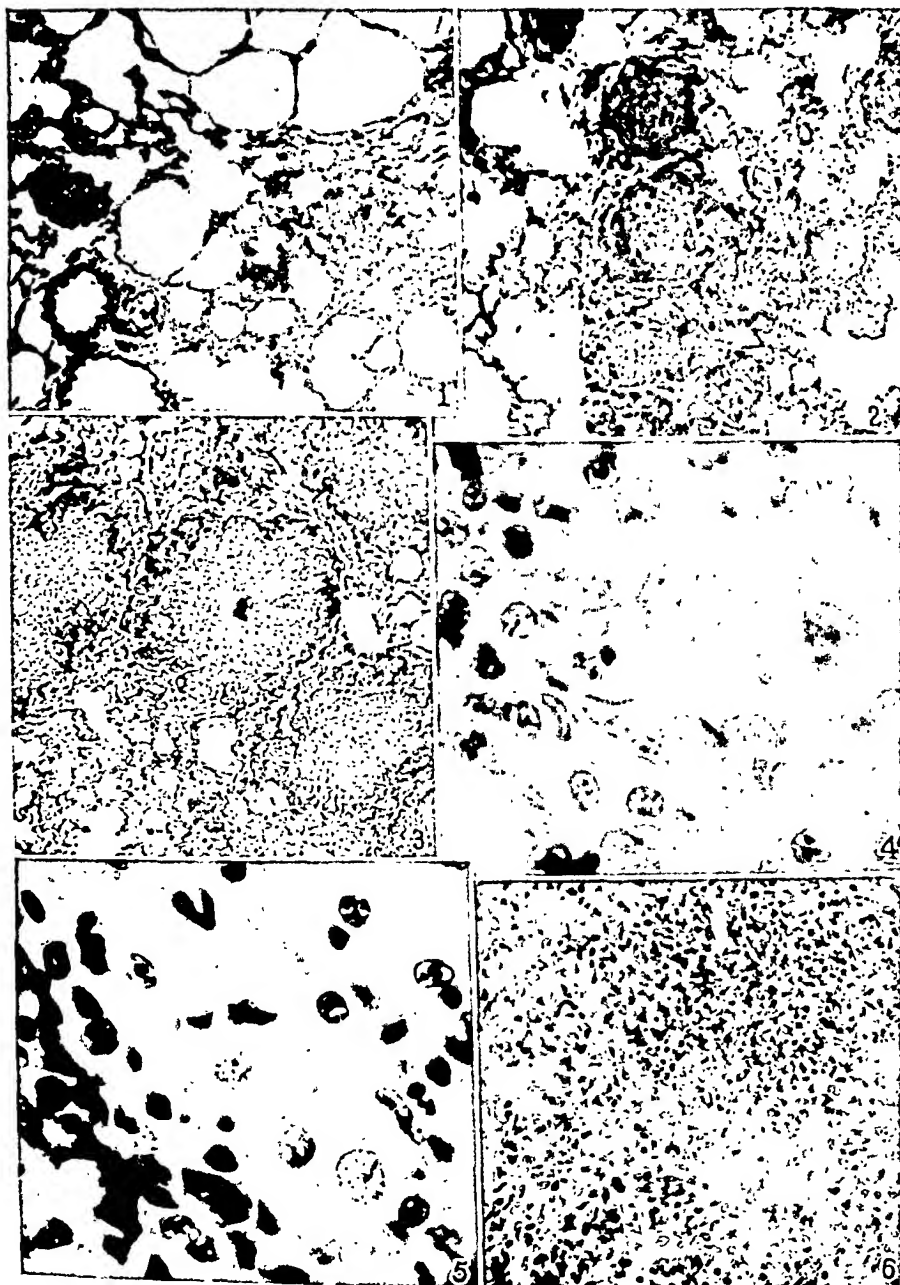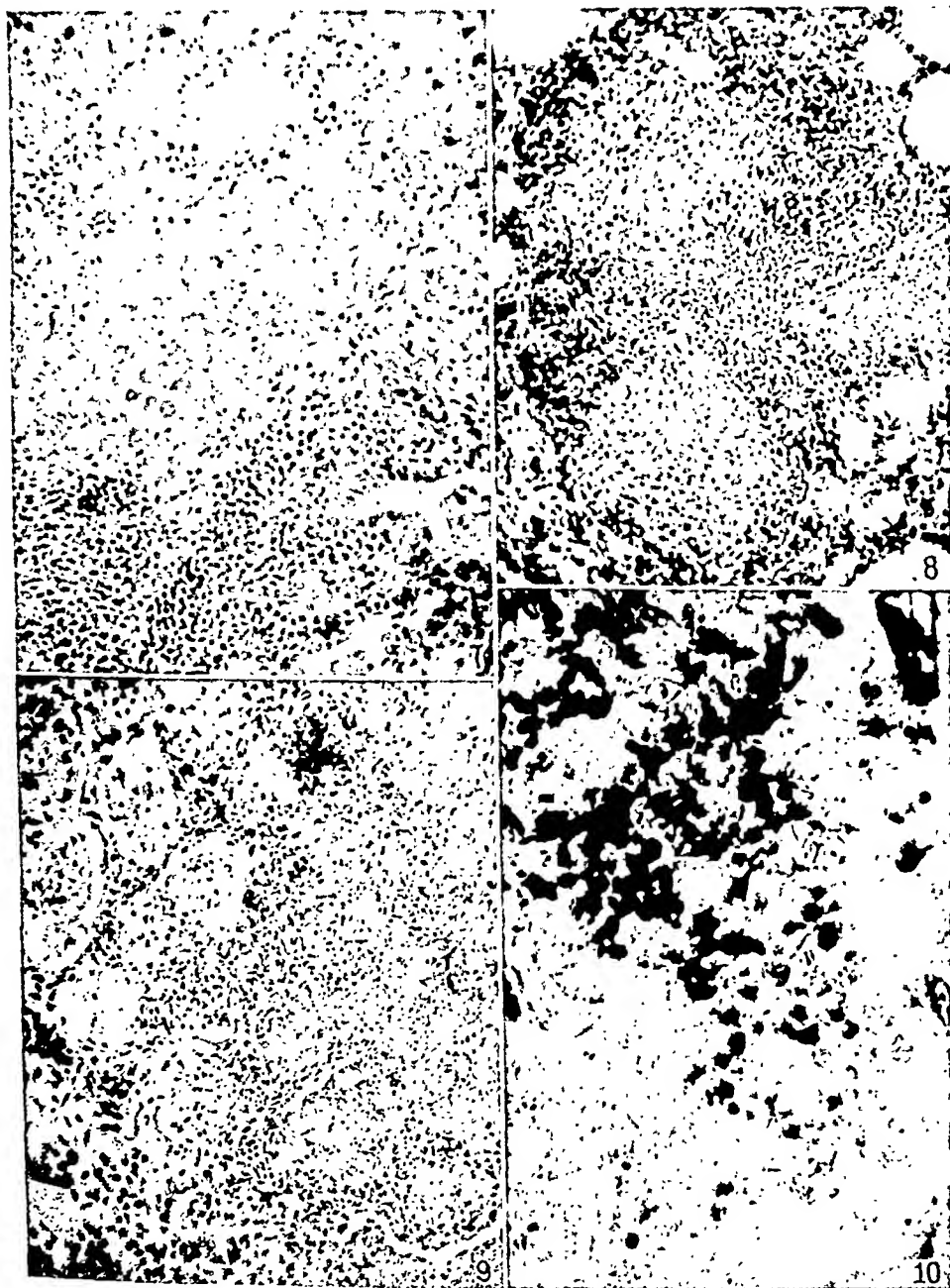
(Lurie: Tubercle bacilli)

(Lurie: Tubercle bacilli)

PLATE 2

(Lurie: Tubercle bacilli)

PLATE 3
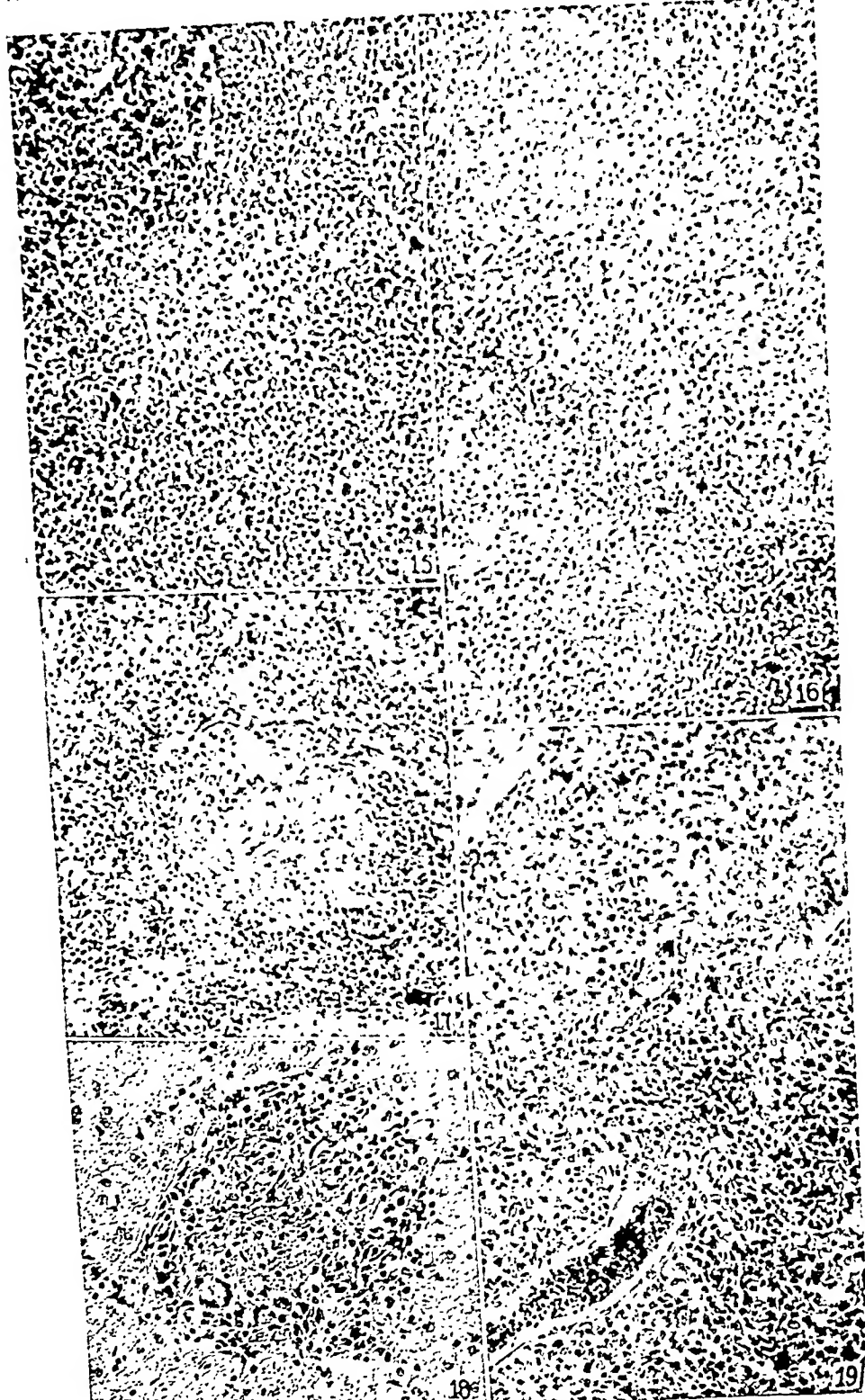
(Lurie: Tubercle bacilli)

THE IDENTITY OF THE MECHANISMS OF TYPE-SPECIFIC AGGLUTININ AND PRECIPITIN REACTIONS WITH PNEUMOCOCCUS

By THOMAS FRANCIS, Jr., M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

In the course of some experiments, the serum obtained from a rabbit actively immunized against Type II Pneumococcus was injected intravenously into a normal rabbit. Before injection the serum had been shown to contain type-specific agglutinins for the homologous organism and precipitins for the type-specific polysaccharide derived from organisms of the same type. When the serum of the passively immunized animal was tested 24 hours later, type-specific agglutinins were present but no visible precipitin reaction with the polysaccharide could be demonstrated. Since the haptens are known to produce inhibition phenomena in serological reactions (the type-specific polysaccharides react serologically as haptens), it was conceivable that in the precipitin tests soluble specific substance (S-free) and antibody had combined without producing precipitation. To test this hypothesis, equal amounts of a suspension of homologous heat-killed pneumococci were added to each of a series of tubes which contained equal amounts of immune serum and varying dilutions of the type-specific polysaccharide. In the tubes containing the highest concentration of type-specific polysaccharide little or no agglutination occurred, whereas in the presence of weak concentrations of soluble specific substance the degree of agglutination approached that present in the tubes containing only immune serum.

The specific capsular polysaccharides are known to be the components of the respective types of Pneumococcus which determine their type specificity. These carbohydrates have been obtained by chemical methods in a purified state, completely separated from the bacterial cell. In this state they are readily soluble and react sero-

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logically in the precipitin reaction with homologous immune sera. The observations just described indicated that soluble specific substance may exert a definite influence upon the type-specific agglutination reaction. The subsequent work was designed, therefore, to ascertain what this influence might be and, if possible, to correlate the mechanisms of the precipitin and agglutinin reactions.

Materials

1. Type-Specific Polysaccharides.—These were obtained from pneumococci by the method of Heidelberger and Avery (1). The dilutions were made in physiological salt solution.

2. Immune Sera.—Immune rabbit serum was obtained from rabbits immunized by the method of Cole and Moore (2). Immune horse serum was obtained from the New York State Board of Health Laboratories through the courtesy of Dr. A. B. Wadsworth. In general, rabbit serum was found more satisfactory than horse serum in the type of experiment here described, since the titer of antibodies in the former is generally not so high and sharper end-points may be obtained.

EXPERIMENTAL

Experiment 1.—To 5 cc. of antipneumococcus Type I horse serum were added the bacteria recovered by centrifugation from 50 cc. of a Type I pneumococcus culture suspended in 5 cc. of physiological saline. The mixture was placed in a water bath at 37°C. for 2 hours—then in the ice box overnight. The next day the tube was centrifugated and the agglutinated material removed. This mass was mixed with 9.5 cc. of Type I S so as to produce a final concentration of 1:200,000 of the latter. The tube was shaken at 15 minute intervals for 2 hours and then placed in the ice box. After 24 hours the tube was centrifugated and the supernatant fluid removed by pipette. The amount of unbound S in the supernatant fluid was determined. This was done by titration with immune serum, comparing the results with those obtained with known amounts of S. If no change in the concentration of S in the fluid had been produced by exposing it to the agglutinated material the end-point should be the same as that of the control. In fact, however, the control material reacted in a dilution of 1:1,600,000 while the S in the supernatant reacted in a dilution equivalent to only 1:400,000, a decrease to one-quarter the amount originally present.

Let us assume, as suggested by Avery and Heidelberger (3), that type-specific agglutination is produced by the union of antibody with the capsular carbohydrate at the surface of the cell (S-cellular). On this basis the probable explanation of Experiment 1 is that when pneumococci, in numbers insufficient to absorb the serum completely,

are specifically agglutinated, an excess of antibody is present in the combination. This excess can then unite with additional free S forming a complex of antibody with free and cellular S. Since the amount of antibody is constant, the ratio of antibody to S is smaller in the second phase than in the primary agglutination.

In the precipitin reaction an excess of precipitinogen added to the precipitate causes the precipitate to dissolve. To clarify the relation of the type-specific agglutinin and precipitin reactions, therefore, it is necessary to determine the effect of an excess of free S upon the combination of antibody and cellular S in the agglutination reaction.

Experiment 2.—To 0.5 cc. of antipneumococcus Type II rabbit serum was added 0.5 cc. of a concentrated suspension of Type II Pneumococcus. Prompt agglutination occurred and a firm disc formed. Following incubation for 2 hours at 37°C., 0.5 cc. of a 1:200 dilution of Type II soluble specific substance was added. The mixture was agitated and placed in the water bath. After 45 minutes agglutination was no longer detectable but the organisms had become redispersed into their original, suspended state.

The experiment was repeated in a modified manner.

After agglutination had occurred, as above, the disc was removed, washed gently with physiological saline once, and mixed with 0.5 cc. of 1:200 Type II soluble specific substance. The agglutinated disc became redispersed. The organisms were then centrifuged from their state of suspension and resuspended in physiological saline, Type I serum, and Type II serum, respectively. That the organisms were not rendered spontaneously agglutinable by the original agglutination was shown by the fact that they did not agglutinate in the saline or Type I serum. Nor had they lost their agglutinability since they were promptly agglutinated by the Type II serum. The supernatant fluid after centrifugation of the organisms gave a well marked reaction with Type II serum showing that an excess of free S was present.

By the above procedures it was clearly demonstrated that an excess of free S produces a prompt and complete reversal of the agglutinin reaction. With the return of the agglutinated bacteria to a state of suspension they are again specifically agglutinable. Two interpretations of the mechanism of the experimental results suggest themselves. The first is that the antibody originally combined with the cellular S is released from that combination and bound in turn by the free S. The other and more consistent explanation is that the combination of

antibody with cellular S at the surface of the cell forms a soluble product in the presence of the excess free S. In the latter instance, however, only a small amount of the capsular polysaccharide is removed from the cell since it regains a normal agglutinability.

In the preliminary experiments it was noted that an excess of free S prevented agglutination and presumably combined with the entire amount of antibody. The following experiment was designed to show that no detectable antibody is bound by cellular S under these conditions, but rather that it is in firm combination with the free soluble specific substance.

Experiment 3.—Pooled antipneumococcus Type II rabbit serum was used. The agglutinin titer was positive in 1:128 dilution; the precipitin titer was + with 1:800,000 dilution of Type II soluble specific substance; and the serum when diluted above 1:20 gave no precipitin reaction with an optimal concentration of soluble specific substance. To 0.2 cc. of serum was added 0.3 cc. of physiological saline and 0.5 cc. of a 1:200 dilution of Type II soluble specific substance. No precipitation occurred after 2 hours incubation and overnight storage in the ice box. To the tube was added 1 cc. of a concentrated heat-killed suspension of Type II pneumococci. The organisms were not agglutinated. In the control tubes, to which no precipitinogen had been added, the addition of organisms resulted in the formation of a typical firm disc. That the agglutinability of the organisms was unchanged after the treatment was shown by removing them from the mixture by centrifugation and resuspending them in saline, Type I and Type II sera. Agglutination occurred only in Type II serum.

The supernatant liquid from which the organisms were removed still contained bound antibody and free S. To show that the antibody had been bound by the free S, the material was diluted so that the serum was in a concentration (1:80) in which untreated serum would cause agglutination but would no longer give a precipitin reaction. To this mixture bacterial suspension was added but no agglutination occurred, nor had dilution of the mixture caused any precipitation.

The explanation of the results of Experiment 3 appears to be that in the presence of an excess of free S the entire mass of antibody combines with it to form a soluble product. Consequently little or no antibody combines with the S of the cell body. As a result, the bacteria are in an unaltered state of reactivity. That is, if an inhibition zone is produced by the free S in the type-specific precipitin reaction, the antibody is no longer capable of agglutinating type-specific organisms when they are introduced into the reaction mixture.

DISCUSSION

Heidelberger and Kendall (4) have suggested that the precipitin reaction follows certain rules which may be expressed in terms of the laws of mass action. In the study they employed the specific capsular polysaccharide of Type III Pneumococcus and an antibody solution derived from an homologous immune serum. Their results and interpretations are briefly as follows:

1. When the smallest amount of type-specific soluble substance (S) capable of producing a precipitate is added to antibody (A), the reaction takes the form

$$A + S \rightleftharpoons A\,S,$$
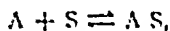
in which the ratio of antibody to S in the precipitate is about 120:1.

2. The precipitate in Equation 1 is capable of reacting with more S to a point at which both components are in equilibrium in solution and the composition of the precipitate is approximately 60:1.

$$A\,S + S \rightleftharpoons A\,S_2.$$
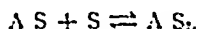
3. With the addition of slightly more S, the point of maximal precipitation is reached, and with further continued addition of S the precipitate gradually redissolves. This is assumed to be associated with the formation of a new combination of A and S which is soluble.

$$A\,S_2 + S \rightleftharpoons A\,S_3.$$
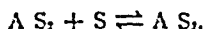
In the three equations the proportions of S combined with A vary as 1:2:3.

Although accurate quantitative estimations were not made, it can be seen that the reaction of type-specific agglutination follows, in general, the principles given by Heidelberger and Kendall for the precipitin reaction.

In Experiment 1 Equations 1 and 2 are realized. When bacteria, in an amount fractional of that required for complete absorption of the immune serum, are added to immune serum, a type-specific agglutination occurs. Substituting in Equation 1 of Heidelberger and Kendall, we derive

I. $A + S$ (cellular) $\rightleftharpoons A\,S$ (cellular).
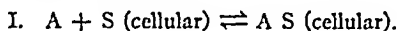
The antibody in the agglutinated mass is able to combine with additional S as shown by the fact that when more free S is added to the agglutinated material some of the S is bound. Heidelberger and Kendall have shown that this is not a simple adsorption. Hence,

II. $A\,S$ (cellular) $+ S$ (free) $\rightleftharpoons A\,S_2$ (cellular and free).
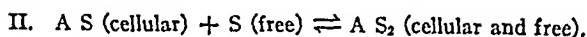
In Experiment 2 the combined reagents may exist either in the form of A S or A S$_2$. In either case, the addition of sufficient free S causes the equilibrium to swing in the direction of Equation III. Under these conditions the antibody forms a soluble compound with S and the agglutinated organisms are redispersed to their original state of suspension.

III.   (a) A S (cellular) + 2 S (free) $\rightleftharpoons$ A S$_3$—agglutination redispersed

or

III.   (b) A S$_2$ (cellular) + S (free) $\rightleftharpoons$ A S$_3$—agglutination redispersed.
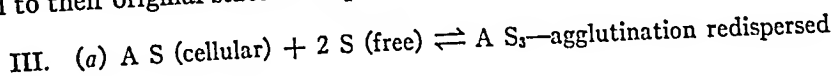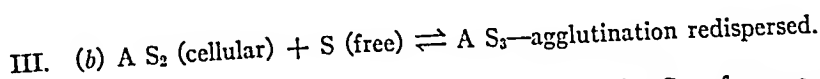
In this instance the antibody is dissociated from the cellular S or forms a soluble combination with the free S. But only a small amount of the cellular S is removed from the cells in this process for they can be agglutinated again in a normal manner with homologous immune serum.

In Experiment 3 the antibody is bound by free S to form A S$_3$, which is soluble. When organisms are subsequently added they are not agglutinated but, removed from the reaction mixture, they are still specifically agglutinable.

IV.   A S$_3$ (free) + S (cellular) $\rightarrow$ no agglutination.
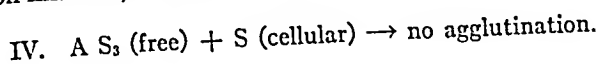
The zone phenomenon encountered in the present instances differs from that usually met with in agglutination reactions. In the agglutination reaction the prozone usually occurs in the presence of an excess of antibody. In the present experiments agglutination is inhibited by an excess of S, the relations usually present in the inhibition of precipitation.

It has previously been indicated that precipitation of a specific soluble antigen may take the form of agglutination in the presence of particulate matter. Nicolle (5) and Arkwright (6) demonstrated that by suspending particulate matter or heterologous bacteria in the soluble precipitinogen of B. typhosus, agglutination of the suspended material was produced by antityphoid serum. Jones (7) suspended collodion particles in solutions of antigens and then removed, by washing, all antigen but that adsorbed by the collodion. When the particulate matter was resuspended and added to serum of animals immunized to the antigen, agglutination occurred. The same reactions were obtained when bacteria were treated with non-bacterial antigens. Mudd and his associates (8) have recently confirmed Jones' results. White (9) has shown that saline extracts of organisms of the Salmonella group, or "carbohydrate-containing hap-

tens," prepared by treating the organisms with alkali, when added to homologous immune serum were able to inhibit the action of the somatic agglutinins for bacterial cells subsequently added to the mixture. No influence was exerted upon the flagellar agglutinins. In the present experiments the same specifically reactive substance is present in both the precipitation and agglutination reactions. In the latter it is part of the bacterial cell; in the former it is chemically pure and separate from the cell. In both instances the reactions, whether agglutination or precipitation, appear to be controlled by the same laws.

SUMMARY

The experimental results which have been described demonstrate the following facts:

1. In the type-specific agglutination reaction, when the organisms are not present in sufficient numbers to absorb completely all the antibodies from the serum, more antibody is bound by cellular S than is required for the process of agglutination.

2. The excess of antibody thus bound can then unite with additional amounts of the specific substance when this is added in soluble form to the agglutinated material.

3. If an excess of the free S is added to an agglutinated mass of antibody and bacteria, the organisms are redispersed and in the suspended state are again specifically agglutinable.

4. When a solution of the specific polysaccharide is added in excess to an homologous immune serum, a prozone is created in which precipitation is inhibited; moreover, if, at this point, type-specific pneumococci are added to the mixture, inhibition of agglutination also occurs.

5. The reactive substance in the type-specific agglutination and precipitation reactions is the same, i.e., the capsular polysaccharide. In the former instance it is combined in the bacterial cell; in the latter, it is in a soluble, chemically purified state and entirely separate from the body of the cell.

CONCLUSION

The results obtained in the agglutination reaction conform qualitatively with the principles described by Heidelberger and Kendall

for the phenomenon of precipitation.   They indicate that the essential mechanism of the two reactions is identical and that the active reagents are the same in both.

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PROTECTION OF ADRENALECTOMIZED ANIMALS AGAINST BACTERIAL INTOXICATION BY AN EXTRACT OF THE ADRENAL CORTEX*

By FRANK A. HARTMAN, Ph.D., AND W. J. MERLE SCOTT, M.D.

(From the Department of Physiology, the University of Buffalo, Buffalo, and the Department of Surgery, the University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.)

In 1924, Scott (1) demonstrated the protective power of the adrenal cortex against intoxication with pyogenic bacteria, both staphylococcus and streptococcus. Because of the probable importance of such non-specific resistance in human infections, repeated attempts to obtain a cortical extract which would increase the lowered resistance of adrenalectomized animals, were made at that time but without success. Marine and his coworkers have amply demonstrated this increased susceptibility of adrenal-deficient animals to bacterial intoxication (2–4), Jaffe in their laboratory being successful in replacing this function of the normal adrenal cortex by that of autoplastic transplants. Recent progress with extraction methods has produced cortical extracts of proven efficiency in maintaining life in the presence of a total adrenal deficiency (5, 6).

Recently Scott and Bradford (7) found a slight increase in the resistance to bacterial intoxication conferred by the administration of one such extract, that of Swingle and Pfiffner (6). The chief difficulty encountered was that no satisfactory criterion of adequate substitution was available. Hartman and Thorn (8) have introduced a test for the adequacy of substitution by the cortical hormone after adrenalectomy in rats. The present study was undertaken therefore to determine whether an extract of the adrenal cortex could adequately protect adrenal-deficient animals from bacterial intoxication.[1]

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animals in Group B (saline-injected) were dead and all in Group A (extract-injected) were alive. Four of Group A died later, but ten of the fourteen in this group entirely recovered from the effects of intoxication with typhoid bacilli and survived the test. 6 hours after the first injection of typhoid vaccine and shortly after the death of the last control animals two of the extract-injected animals appeared very ill. They were each given an extra 0.5 cc. of cortin. The condition of one improved at the end of 1 hour while the other one died some time during the next 11 hours. A third cortin-injected rat became prostrate and went into convulsions 7 hours after the first cortin injection. 1 cc. of cortical extract was given intravenously but respirations did not return. Two others died during the night (11+ hours after the typhoid administration). One of these apparently had a respiratory infection. He had failed to regain his growth curve under the administration of cortin and his lungs at autopsy showed mottled areas not found in the other three animals of Group A that died. Beginning 10 hours after the injection of the typhoid vaccine, the surviving ten rats of Group A (cortin-injected, all saline-injected animals having died) received no injections overnight, that is to say for a period of 7 hours. It was during this time that three out of the four cortin-injected rats died. It is possible that if the animals had been closely watched during this interval and injected at critical times some would have survived. This is borne out by the outcome in the rat mentioned above that recovered from a critical condition after an extra injection. In spite of this overnight intermission, ten of the fourteen rats in Group A (cortin-injected) survived. These were given cortin twice daily until their growth curves indicated complete recovery, when the injections were discontinued. After discontinuing the extract, thereafter the growth rate markedly diminished. 16 days after the typhoid injection they were killed and a careful search made for evidence of cortical tissue. This was found in only one animal, which showed a mass of tissue about 1.5 mm. in diameter, proven histologically to be made up of cortical cells. No cortical tissue had been found in the animals which had died as the result of typhoid injections.

Discussion.—This experiment seemed to us conclusive evidence that the extract of the adrenal cortex which was used afforded a definite degree of protection against bacterial intoxication in the diminished resistance of adrenal insufficiency. This we consider is important to prove conclusively, as we believe that the hormone of the adrenal cortex plays a very important rôle in human pyogenic infections. In the first experiment, killed typhoid organisms were used because a somewhat better standardized intoxicant can be obtained with them than with other bacteria. In order to make certain that the same protection was afforded against intoxication with the ordinary pyogenic organisms, another experiment was tried.

Experiment II

Forty rats were available that had been used in estimating the potency of various lots of cortical extract though they were now no longer receiving it. They were divided into two corresponding groups of twenty animals each, and kept under the standard conditions of the first experiment except that their weight curves were not followed. Every animal in both groups was injected daily with killed *Staphylococcus aureus* in ascending dosage, beginning with 2 billion organisms on each of the first 2 days, increasing to 5 billion on the 5th, 6th and 7th days, jump-

CHART 2. Influence of daily injection of killed *Staphylococcus aureus* on survival of adrenalectomized rats.

ing to 10 billion on the 8th and 9th days and increasing by 2 billion daily to 16 billion organisms on the 12th and 13th days. One group received cortin injections subcutaneously, 0.5 cc. twice a day, while the animals in the other group were given a corresponding volume of isotonic sodium chloride solution at the same intervals. Chart 2 shows graphically the results of this experiment. Six animals in the control group (receiving salt solution) died on the 2nd day and by the 9th day twelve animals, or 60 per cent of this group, had died while none of the animals receiving cortin had as yet succumbed. The first animals of the cortin group died on the 9th day having received by that time 46 billion killed staphylococci in this interval. By the 11th day of the experiment, sixteen of the twenty control animals (80 per cent) had succumbed while only four of the twenty cortin-treated

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rats (20 per cent) had done so. With the progressively increased dosage of dead bacteria the protection afforded to the animals in the cortin group by the limited amount of extract given was overcome in most cases. On the 13th day, the remaining four animals in the control group and eleven of the rats in the cortin group died. Two of the four control animals surviving to the 13th day but dying on that day were found on autopsy to have small accessory adrenals. The other two had no masses large enough to see grossly. That they had microscopic accessories is possible because they both gained in weight, one showing a considerable gain. None of the eleven animals in the cortin group dying on the 13th day showed any accessories. Three cortin-injected animals survived the entire experiment and were sacrificed. One of them showed an accessory gland, the other two did not.

Discussion.—The effect of the bacterial intoxication was so different in the two groups that it is hardly necessary to treat the results mathematically. The three animals that showed gross adrenal cortical tissue were excluded from statistical analysis. The average fatal dose was 34 billion staphylococci for the control animals compared to 95 billion for those treated with extract. Furthermore, two cortin-injected rats survived and eleven of them succumbed only after they had received more than 100 billion killed staphylococci, while none of the saline-injected rats survived and all but two succumbed to 58 billion organisms or less. Even the two saline-injected rats in which cortical tissue was found died after receiving 103 billion staphylococci in 13 days. This fact is a functional verification of a previous histological observation made by one of us, *viz.*, that repeated injections of killed staphylococci caused an exhaustion of cortical accessories (1).

It is our impression that we could have afforded still more protection to the animals in Group A by increasing the dosage of cortin. However, we purposely restricted the extract to the amount used because we desired to show how much protection can be obtained from a limited dose. This second experiment shows the protection in adrenal insufficiency that can be obtained for chronic pyogenic intoxication. It is our belief that all severe pyogenic infections with constitutional effects lasting over a period of 2 to 3 weeks may be associated with inadequate production of cortin. Further work on this hypothesis is in progress.

At the same time that we were studying the influence of an adrenal cortical extract on the diminished resistance of adrenal deficient animals this question was being investigated independently by Perla

and Marmorston-Gottesman in Marine's laboratory, using the same type of extract (10). Their results are in complete agreement with ours.

CONCLUSIONS

1. The resistance of adrenal-deficient rats to bacterial intoxication has been significantly increased by an extract of the adrenal cortex.

2. This is shown both for acute intoxication with killed *Bacillus typhosus* and for chronic intoxication with killed *Staphylococcus aureus*.

3. During the height of the bacterial intoxication relatively large amounts of the cortical hormone are apparently required to maintain the animals.

4. It is considered probable that a human pyogenic infection imposes a severe load upon the adrenal cortex.

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THE EPIDEMIOLOGY OF FOWL CHOLERA

VI. THE SPREAD OF EPIDEMIC AND ENDEMIC STRAINS OF PASTEURELLA AVICIDA IN LABORATORY POPULATIONS OF NORMAL FOWL

BY I. W. PRITCHETT, Sc.D., AND T. P. HUGHES, Ph.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

In previous studies on spontaneous fowl cholera in commercial poultry flocks, it was noted that the epidemic form of infection was usually associated with strains of *Pasteurella avicida* which appeared to be relatively highly virulent and unable to survive in the tissues of the host, while the endemic infection was associated with strains of low virulence and high vegetative capacity (1). The present experiments were made to test these relationships under the more controlled conditions of the laboratory, and in addition to determine whether "laboratory" variants of the epidemic strains were similar to the endemic strains in their capacity to survive and spread in their native host.

Technique

The young birds used in these tests were White Leghorns from a single inbred flock. The chicks were received in the laboratory in batches of 100 to 200 when 1 day old and were raised in strictly isolated quarters out of contact with other birds. They were free of infectious disease and exposure to *P. avicida*. The older birds used in Experiment 3 were White Leghorns of an unselected farm stock.

The strains of *P. avicida* employed have been described previously (2). The "Kansas" strain was obtained from a spontaneous epidemic of fowl cholera; Strain 773 was obtained from a flock in which fowl cholera was endemic. *P. avicida* was administered to the test birds in a manner calculated to simulate natural conditions and at the same time be relatively quantitative, by instilling into the nasal cleft of each individual a uniform number of the bacteria from an 18–24 hour blood broth culture. Carrier tests were made by passing a sterile cotton covered swab over the surface of the nasal cleft of the bird and subsequently streaking it over the surface of a freshly prepared blood agar plate. Birds dying in the course of the experiments were autopsied and cultured for the presence of

P. avicida. These various procedures have been fully described in earlier publications (1,2).

Epidemic Strains

Experiment 1.—March 12, 1928. Eight chicks, aged 1 month, were each given intranasally 0.2 cc. broth culture of *P. avicida* "Kansas" and placed in a cage measuring 12 x 16 x 12 inches. Eight similar uninoculated birds were added to the group as "contacts." Examinations for carriers of *P. avicida* were made 1, 3, 5, 24, and 30 hours, and 2, 3, 4, and 7 days later.

TABLE I

Virulence and Spread of an Epidemic Strain of P. avicida

Test animals	Hrs.					Days				Condition of animal
	1	3	5	24	30	2	3	4	7	
Inoculated No. 1	+	+	+	+	+	+				Died
" " 2	+	+	+	+	+	0	0	0	0	Well
" " 3	+	+	+	+	+	0	0	0	0	"
" " 4	+	+	+	+						Died
" " 5	+	+	+	+	+	0	0	0	0	Well
" " 6	+	+	+	+	+	+	0	0	0	"
" " 7	+	+	+	+	+	+				Died
" " 8	+	+	+	+						"
"Contact" Nos. 1 to 8	0	0	0	0	0	0	0	0	0	Well

+ = *P. avicida* present.   0 = *P. avicida* absent.

The results of this experiment are given in Table I. 50 per cent of the inoculated birds were dead of fowl cholera within 3 days—a figure which agrees with previous data on the virulence of the "Kansas" strain (2). The survivors, however, showed no signs of infection and no evidence of carrying the organisms in the nasal cleft after the 2nd day. The contacts remained healthy and did not become carriers.

This test was repeated March, 1928, with similar results.

Experiment 2.—February 8, 1929. Twenty chicks, aged 1 month, were each given 0.2 cc. of *P. avicida* "Kansas" intranasally and placed in a floor pen 4 x 8 feet in dimension. Normal, uninoculated birds were added thereafter at the rate

given intranasally 0.2 cc. of *P. avicida* "Kansas" and placed in a suitable pen. Normal, uninoculated birds were added thereafter for 20 days at the rate of one per day.   Carrier tests were made on the 7th and 18th days.

Table III shows the results of this test.   Sixteen of the inoculated birds died of fowl cholera within 9 days.   On the 7th day, five birds proved to be carriers.   Three of these died of cholera 2 days later and

TABLE IV

Virulence and Spread of an Epidemic Strain of P. avicida

Date	Identification No. of dead chicks	Surviving population	Remarks
2/27/29		40	Birds numbered 1 to 20 inoculated. Birds numbered 21 to 40 added immediately. One normal bird added daily and numbered serially from 41 to 52
2/28/29	7	40	
3/ 1/29	5, 10, 11, 12, 15, 17, 18, 19	33	
3/ 2/29	14	33	
3/ 3/29	2, 20	32	
3/ 4/29		33	Tested for carriers. All negative
3/ 5/29	6	33	
3/ 8/29		36	" " " " "
3/11/29		38	Addition of birds discontinued
3/12/29		38	Seven survivors and 32 contacts given 0.2 cc. *P. avicida* intranasally
3/13/29	21, 23, 26, 31, 45, 46	32	
3/14/29	33, 39	30	
3/15/29	22	29	
3/16/29	26, 34	27	
3/17/29	32, 55	25	
3/18/29	16, 44	23	Experiment terminated

one 9 days later, after a second positive carrier test.   The other survivors and all contacts showed no signs of infection and did not become carriers.

Experiment 4.—February 27, 1929. Twenty chicks, aged 1 month, were each given intranasally 0.2 cc. of the "Kansas" strain of *P. avicida* and placed in a floor pen.   Immediately thereafter twenty normal birds were added and subsequently one bird per day for 12 days. Carrier tests were made on the 5th and 9th days.

On the 10th day, all survivors and contacts were given an intranasal dose of 0.2 cc. of *P. avicida*.

The results of this test are given in Table IV. Thirteen of the twenty originally inoculated birds (65 per cent) died of cholera within 6 days. The remainder, together with the thirty-two contacts, showed no signs of infection and no *P. avicida* when the two carrier tests were made. Subsequent intranasal inoculation of the contacts, however, was followed by a 44 per cent cholera mortality, indicating that they were susceptible to *P. avicida*.

Similar tests with two other epidemic strains of *P. avicida* from different sources gave similar results. It is concluded, therefore, that the epidemic strains of *P. avicida* tested are of relatively high virulence but do not survive in the tissues of the healthy host nor readily spread and survive in contact hosts.

Endemic Strains

Experiment 5.—March 25, 1929. Five chicks, aged 1 month, were each given 0.2 cc. of an 18 hour broth culture of the endemic Strain 773 and placed in a cage measuring 12 x 16 x 12 inches in dimension. Five normal, uninoculated chicks were then added. Carrier tests were made on the 1st, 4th, 5th, 6th, 9th, 10th, 13th, 15th, and 17th days of observation. On the 28th day, all were given intranasally 0.2 cc. of the epidemic "Kansas" strain of *P. avicida*.

The results of this test, given in Table V, differ sharply from those of the preceding tests. In the first place, none of the inoculated birds died, in the second place, all became carriers, and in the third place, all the normal contact birds remained healthy but became carriers. Finally, upon exposure to the epidemic strain, 50 per cent of these contacts succumbed to cholera.

A second similar test gave comparable results and showed that the endemic strain of *P. avicida* was capable of surviving in the nasal cleft for at least 65 days.

Experiment 6.—April 25, 1929. Twenty chicks, 1 month old, were inoculated intranasally with 0.2 cc. of the endemic strain of *P. avicida*, No. 773, and placed in a floor pen. Ten similar uninoculated chicks were added at once and one daily thereafter for 30 days. Carrier tests were made at frequent intervals for 60 days.

All birds remained healthy. *P. avicida* spread to one contact within 6 days and to ten within 4 weeks. In 5 weeks 20 per cent of the inoculated and 31 per cent of the contacts were carriers, and in and 7 weeks 55 per cent and 40 per cent respectively. Other endemic strains tested gave similar results.

From these experiments it is concluded that the endemic strains under observation were of relatively low virulence and high vegetative capacity and ability to spread from host to host.

<div align="center">TABLE V</div>

<div align="center">*Virulence and Spread of an Endemic Strain of P. avicida*</div>

Date	Identification No. of dead birds	Identification No. of carriers	Remarks
3/25/29			Birds numbered 1 to 5 inoculated; birds numbered 6 to 10 added immediately
3/26/29		4, 5	
3/29/29		2	
3/30/29		5, 7	
4/ 1/29		1, 2, 3, 6, 10	
4/ 4/29		2, 3, 4, 5, 6, 7, 8, 10	
4/ 5/29		1, 4, 5, 6, 7, 8, 9, 10	
4/ 8/29		1, 4, 5, 6, 8, 9, 10	
4/10/29		4, 5, 6, 7, 8, 9, 10	
4/12/29		1, 2, 3, 4, 5, 6, 7, 8, 9, 10	
4/23/29			All inoculated intranasally with 0.2 cc. "Kansas"
4/25/29	3, 4, 7, 8, 9, 10		
4/29/29		1 (Endemic strain)	
4/30/29			Experiment terminated

"*Laboratory*" *Variants from the Epidemic Strain.*—Epidemic strains of *P. avicida* will, when cultivated in broth or on agar under aerobic conditions, undergo dissociation. Details of this process have been described previously (3). Certain of these variants bear a close resemblance to the endemic strains and insofar as can be determined by bacteriological and immunological tests, are identical. It seemed desirable, however, to submit them to an epidemiological test, that is, to determine whether or not they resembled the endemic strains in

being of low virulence and high capacity to survive and spread in fowl populations. Tests similar to those described above were run with four variants obtained from the "Kansas" strain. In no instance did the variants kill and, with a single exception in which two contacts were carriers for 1 day, in no instance did they spread to normal contacts. They survived in the nasal clefts of the inoculated birds for periods less than 2 weeks. Thus the laboratory variants differed fundamentally from the endemic strains in not surviving in the host or spreading to contacts.

DISCUSSION

The distinctive parasitic characteristics of epidemic and endemic strains of *P. avicida* brought out by these experiments are not limited to these organisms alone. The same relationships have been shown to exist in the case of epidemic and endemic strains of *P. lepiseptica* (4), and epidemic strains of mouse Friedländer and endemic strains of mouse typhoid (*B. enteritidis*) bacilli (5). Similar distinctions in parasitic behavior probably exist among the various types of pneumococci (6). It appears, therefore, that these inherent, relatively stable characteristics of parasites which are demonstrable experimentally and under natural conditions are common to many specific agents and that they determine largely the severity and spread of infection in a previously unexposed population.

The failure of epidemic strains of *P. avicida* to survive in nature and to spread among specially bred chickens in the experiments just described is not understood. Possibly they require hosts of abnormally low resistance; possibly they require the presence of some other agent which as yet has not been discovered.

Variants of epidemic strains occurring under laboratory conditions, although indistinguishable from endemic strains according to bacteriological and immunological tests, were shown to be quite different by the epidemiological test. Care should be exercised, therefore, in assuming that laboratory strains necessarily behave in a similar manner to parasites under natural conditions.

CONCLUSIONS

1. Strains of *P. avicida* from "spontaneous epidemics" of fowl cholera, when introduced intranasally in fixed doses into specially bred chickens,

induced fatal fowl cholera in about 40 per cent but did not survive in the nasal clefts of resistant birds nor spread to normal contacts.

2. Strains of *P. avicida* from "spontaneous endemics" of fowl cholera, when introduced similarly into chickens, failed tò kill but did survive in the nasal clefts of inoculated birds and spread readily to normal contacts.

3. "Laboratory" variants of the epidemic strains of *P. avicida* failed to kill or survive in the test birds and did not spread to contacts.

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SERUM SICKNESS IN RABBITS

II. Precipitinogen and Precipitins in Relation to Appearance of the Reaction

By LLOYD JONES, Ph.D., and MOYER S. FLEISHER, M.D.

(From the Department of Bacteriology and Hygiene, St. Louis University School of Medicine, St. Louis)

From the earliest days of the recognition of the syndrome of serum sickness in man, studies have been carried out in an effort to discover what relationship might exist between serum sickness and the demonstrable antigens and antibodies present in the affected individual. The evidence which has been gathered suggests the possibility of such relationship, but there exist a number of observations which apparently militate against sweeping or definite statements concerning this relationship.

We (1) have noted in rabbits the appearance of a reaction on the ears occurring usually 5 to 7 days after a single injection of a quantity of horse serum greater than 5 cc. pro kilo. The reaction is characterized by the presence of a more or less confluent erythema which may be morbilliform or scarlatinal, involving especially the lower third of the ears but frequently extending over the lower three-quarters of the ear. Frequently there is associated with this a more or less marked edema again especially evident or marked in the lower third of the ear. Such reactions were noted in 69 per cent of 103 animals. Limited reactions of erythema or edema were not considered as positive reactions since they corresponded in part with phenomena which were noted on the ears of normal animals. In 147 normal animals observed over periods of 2 weeks or longer, scattered discrete erythematous areas were noted in many cases and areas of edema involving the upper and middle portion of the ear were noted in thirty-five animals. The reactions of serum sickness as seen on the ears in rabbits differ distinctly from the reactions in normal animals both as to character and location.

induced fatal fowl cholera in about 40 per cent but did not survive in the nasal clefts of resistant birds nor spread to normal contacts.

2. Strains of *P. avicida* from "spontaneous endemics" of fowl cholera, when introduced similarly into chickens, failed to kill but did survive in the nasal clefts of inoculated birds and spread readily to normal contacts.

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It was naturally of interest to study in rabbits affected with serum sickness both the development of antibodies (precipitins) and the disappearance of the injected antigen with the view of determining what relationship might be demonstrable under these experimental conditions.

Method

In twenty-seven rabbits treated as described in the above mentioned earlier paper the precipitin production and the disappearance of horse serum from the circulating blood was studied.   In a few animals tests for precipitins against horse serum were made before the injections, and in all these animals no precipitins could be demonstrated.   In a number of animals blood was taken on the 1st or 2nd day after injection of horse serum, but in others blood was taken first on the 3rd or 4th day after injection.   As a rule the examinations were made at 2 day intervals through the 8th or 9th day, and in a number of animals the examinations were made at the 10th, 11th, 12th, 14th, 15th or 18th days after injection.   Since the majority of the animals showed serum sickness within the period including the 5th to 8th days, it was particularly the results observed within this time that were of interest.

Blood was taken from the heart for all tests.   It was not possible to use the ear veins for obtaining specimens in view of the fact that the reactions of serum sickness were apparent in the ears.   In titrating for the precipitins, the customary technic of using constant quantities of serum (in this case the serum of the rabbit under observation) and diminishing quantities of the horse serum antigen was followed: the readings of the results are therefore expressed in dilutions of the antigen which gave positive reactions with the rabbit serum.   In titrating for the antigen (horse serum) found in the blood of rabbits used in the experiments, serum of rabbits which had been immunized against horse serum was used in a constant quantity, and the serum of the experimental animals diluted in the same gradations as used for the antigen in the precipitin tests, was added.   The dilutions of the precipitinogens ran the scale from 1:10 to 1:20,960, the dilution being doubled in each successive tube.   In all precipitinogen titrations, the antibody (serum of immunized rabbits) was diluted 1:5.   In all the tests a parallel titration with known anti-horse precipitin serum and horse antigen was run.

Of fifteen rabbits injected intravenously, eight developed serum sickness and of twelve injected into the subscapular region ten developed serum sickness.   No essential difference as regards either the precipitin or residual precipitinogen curves could be observed between these two groups, so that in further discussion we may consider together the two groups which were injected in these two different manners.

There are various possibilities which one might expect as demonstrative of the relationship of the precipitins or the content of precipitinogen to the reaction of serum sickness. The precipitins might show a distinct rise or increase at the time of the reaction, or the precipitins might show a suppression or delay in appearance in reacting animals as compared with non-reacting but injected animals. Or the precipitins, if previously present, might show a fall in titre at the time of reaction. Considering the precipitinogen, we might seek for evidence of a rapid fall of this substance in the blood of reacting animals, or an unusually slow disappearance of the injected antigen. There might be evident after, rather than before or during the period of reaction, changes in the content of either precipitin or precipitinogen in the blood of the reacting animals. Or finally, there might be observable in the reacting animals certain relations between the two agents which differed from those seen in non-reacting animals.

It must, however, be stated that when we compare the curves of either precipitins or precipitinogen in non-reacting animals with those occurring in the reacting rabbits it is impossible to demonstrate any regularly occurring differences between the two groups or to note in the rabbits with serum sickness any consistent relationship between either precipitin or precipitinogen and the occurrence of the reactions.

In the nine non-reacting animals we find that in two animals no antibody was demonstrable at 5 days, in two none at 6 days (in no animals were antibodies found at 4 days), in five animals precipitins were demonstrable at 6 days in a titre varying from 1:40 to 1:1280. After the 5th or 6th days there was usually a marked increase in the titre of the antibody, but there appeared to be no regularity in the quantitative increase during the next 2 day period, so that the titre varied between 1:160 and 1:5120 on the 7th and 8th days. On the subsequent days (10th to 18th) there was usually an increase of the titre, in some animals very marked and rapid, in others slower, and in some animals little or no increase in the titre after the 8th day. In a few animals there occurred a drop in the titre after the 8th day, followed by a rise in later periods.

The highest dilution of the serum of the injected non-reacting rabbit in which antigen was demonstrated showed also wide variations in individual animals in the first 5 days, but there is evident within this period a tendency for a fairly rapid fall in the concentration of the horse serum in the blood. In the period from the 5th to 8th days there seems to be quite regularly (with one exception) a slower disappearance of the antigen from the blood stream; a tendency to maintain

TABLE I

Precipitin and Precipitinogen Titre in Blood of Non-Reacting Rabbits Subsequent to Injection of Horse Serum

Rabbit No.	Days after injection..	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
111	Precipitin		0		0		0		640		5,120				5,120				10,240
	Precipitinogen		2,560		640		320		80		160				0				0
112	Precipitin		0		0		80		5,120		5,120				5,120				10,240
	Precipitinogen		1,280		320		320		320		40				0				0
114	Precipitin		0		0	0	0		640		2,560				2,560				2,560
	Precipitinogen		2,560		1,280	160	320		320		40				20				0
115	Precipitin		0		0	0	40		1,280		5,120				1,280				2,560
	Precipitinogen		20,480		1,280	160	320		320		80				20				0
132	Precipitin				0	0	1,280		5,120		5,120		2,560		5,120				
	Precipitinogen				320	160	160		160		80		20		0				
156	Precipitin	0		0				1,280				5,120				10,240			
	Precipitinogen	640		640				320				40				40			
157	Precipitin·	0		0				2,560				20,480				20,480			
	Precipitinogen	640		320				160				10				0			
300	Precipitin				0		80		160				20,480						
	Precipitinogen				1,280		320		160				80						
303	Precipitin				0		320		1,280				5,120						
	Precipitinogen				640		320		320				40						

The figures in the table represent the lowest dilution of antigen giving precipitin reactions and should be read as 1: (the figure given).

a constant level (in one animal there was even an increase between the 5th and 7th days) of the circulating antigen. In later periods the antigen disappears; in four of seven animals examined at 14 or 15 days and in all tested at 18 days (four) there appeared to be no antigen present. The residual antigen in the latter periods, however, varied considerably in the different animals.

It is evident that in the non-reacting animals no regularity can be noted in regard to time relations and titres of either precipitins or precipitinogens.

Considering now the rabbits which showed serum sickness we find essentially the same degree of variation in the curves of the titres of the two agents studied as we have just described above in the non-reacting rabbits. It is apparent that on this basis we can draw no difference.

In one animal (No. 131) serum sickness first appeared on the 3rd day after injection; blood taken on the 4th day showed as in other rabbits, no precipitins; in another rabbit, showing reaction on the 5th day (No. 305), no precipitins were evident on this same day; in a 3rd rabbit showing serum sickness on the 5th day (No. 113) no precipitins were demonstrable on either the 4th or 6th days, and in a fourth rabbit showing serum sickness on the 7th day (No. 154) no precipitins were found on this day.

In several rabbits no precipitins were shown on the day before the appearance of the reaction (Nos. 116, 135, 159, 326, 304) but on the 2nd day of the reaction precipitins varying in titre from 1:160 to 1:640 were evident.

In a number of rabbits (Nos. 160, 162, 321) precipitins were found in titre varying from 1:20 to 1:320 either 1 or 2 days before the appearance of the reactions. From these observations it would seem that the appearance of the reaction cannot be correlated with either the appearance or non-appearance in the blood of precipitins.

We find the precipitin titre on the days of the reactions varying from 0 to 1:2560 and fairly evenly distributed through this range.

On the days subsequent to the reaction, in most cases 1 or 2 days after the last day of evidence of serum sickness, the titre of the precipitins seems to correspond quite well with the titres noted in non-reacting rabbits, and shows neither an unusual increase nor a retardation in the rise usually noted at these days.

In the later periods following the 8th day after injection, the variations in the individual reacting animals are like those noted in the other injected rabbits.

Generally the fall of the precipitinogen in the reacting animals is similar to that noted in other rabbits; a fairly rapid but irregular tendency towards a diminution within the first 5 days; a tendency towards a slower fall between the 5th and 8th days, and a more or less rapid fall towards complete disappearance at 14 to 18 days. Certainly there is no evidence that the antigen disappears more rapidly

in the reacting animals (between the 12th and 15th days) than in the non-reacting ones; the suggestion is rather that the reverse occurs, but probably these differences evident here are within the limits of experimental variations.   In one animal only (No. 159) is there evident any tendency towards a rise of the antigen titre subsequent to and in this case during the appearance of the reaction.   In one case also (No. 113) is there apparent a marked fall of the titre after the appearance of the reaction.   However, similar appearances may be noted at similar periods after injections in non-reacting animals.

It is not possible either to point out any particular or constant relationship existing between the titres of antigen and antibody found in the blood at the time of appearance of serum sickness in these rabbits.   We can find all sorts of relationship; high antigen titre and low antibody titre; low antigen and high antibody, and the titres of the two agents very nearly equal.

DISCUSSION

In one of the earliest publications of von Pirquet and Schick (2) concerning serum sickness they demonstrate certain relations between the appearance of precipitins and the reactions of this phenomenon, and state that precipitins appear after serum sickness has begun.   They are apparently of the opinion that the precipitins never appeared before the symptoms of serum sickness.   In the majority of cases precipitins appeared in the blood on the 8th or 9th day after injection.   Von Pirquet (3) however bases his explanation of the physiology of the reaction of serum sickness and other allergic phenomena largely upon the relationship of antigen and antibody within the affected individual.   Hamburger and Moro (4) who studied the precipitin and precipitinogen in the blood of patients showing serum sickness after injection of Moser's antiscarlatinal serum, state that precipitins appear 2 days after the appearance of serum sickness—namely on the 16th and 14th days respectively.   The antigen disappeared from the blood at about the 23rd day (about 7 days after serum sickness) or sometimes the antigen persisted for 16 or more days after the reaction.   They found that in rabbits injected with 0.5 to 10 cc. of horse serum pro kilo, precipitins appeared at 8 days (none were found at 6 days) after injection and the horse serum tended to disappear at about the 9th day.   These authors did not assume a definite relationship between antigen and antibody on one hand and the reaction on the other hand.   Dehne and Hamburger (5) studying the presence of the antigen by means of titration of the residual tetanus antitoxin in the blood of human beings injected subcutaneously found a slow increase of antitoxin in the blood up to the 2nd or 3rd day—subsequently the antitoxin remained constant up to the 8th day, when there occurred a marked fall the in body disappeared in the 3rd week. These authors, of antitoxin coincide so that

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Wells (9) studying the precipitins in individuals injected with antisera, found no influence of age, sex or weight; he believed, however, that the titre increased irregularly but not constantly with the amount of the injected serum. He found precipitins appearing as early as the 4th day, even in a titre of 1:2560, and often as high as 1:1280 on the 5th day. In one case the precipitin titre fell during serum sickness, rising again after disappearance of the reaction; in two additional cases there appeared a rise of the precipitin titre after the reaction. Wells believed that serum sickness is connected with a union of the precipitin element with some other element in some manner not apparent or understood, and that the disappearance of the symptoms of serum sickness is associated with the liberation of the precipitin.

Longcope and Rackemann (10) studied precipitins, anaphylactins and the appearance of skin sensitiveness in persons injected with various antisera. Sensitiveness to skin reactions could not be correlated with serum sickness, although reactions to the intracutaneously injected horse serum appeared earlier in subjects who developed serum sickness than in those who did not Anaphylactins were demonstrated in eleven of twelve persons developing serum sickness, but in none of those which did not; these antibodies were demonstrated only when serum sickness was well advanced or about to terminate. The precipitins were also demonstrable in the individuals who developed serum sickness but not in injected persons who did not develop the phenomenon. In the persons with serum sickness precipitins did not appear until 3 or 4 days after the onset of serum sickness and then rose rapidly. These authors consider serum sickness to be definitely associable with precipitin and precipitinogen, but assume that reactions in the cells must play an important rôle, due possibly to the presence of precipitins in the cells in the early period.

MacKenzie and Leake (11) studied both precipitin and precipitinogen. In a group of subjects showing severe serum sickness, they found the precipitin rising to the maximum near the time the symptoms disappeared, while the antigen fell with the cessation of symptoms. In a group of non-reacting injected individuals no precipitins or very weakly active ones appeared, and the antigen was retained in the blood stream longer than in the first group. A third group of individuals

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Longcope and Rackemann (10) studied precipitins, anaphylactins and the appearance of skin sensitiveness in persons injected with various antisera. Sensitiveness to skin reactions could not be correlated with serum sickness, although reactions to the intracutaneously injected horse serum appeared earlier in subjects who developed serum sickness than in those who did not Anaphylactins were demonstrated in eleven of twelve persons developing serum sickness, but in none of those which did not; these antibodies were demonstrated only when serum sickness was well advanced or about to terminate. The precipitins were also demonstrable in the individuals who developed serum sickness but not in injected persons who did not develop the phenomenon. In the persons with serum sickness precipitins did not appear until 3 or 4 days after the onset of serum sickness and then rose rapidly. These authors consider serum sickness to be definitely associable with precipitin and precipitinogen, but assume that reactions in the cells must play an important rôle, due possibly to the presence of precipitins in the cells in the early period.

MacKenzie and Leake (11) studied both precipitin and precipitinogen. In a group of subjects showing severe serum sickness, they found the precipitin rising to the maximum near the time the symptoms disappeared, while the antigen fell with the cessation of symptoms. In a group of non-reacting injected individuals no precipitins or very weakly active ones appeared, and the antigen was retained in the blood stream longer than in the first group. A third group of individuals

who showed mild symptoms developed only scanty precipitins, and the antigen disappeared slowly. One individual who developed serum sickness showed a high precipitin titre at the end of the period of serum sickness, but the antigen persisted for a longer period in the blood stream. MacKenzie and Leake, like Longcope and Rackemann and Wells, were therefore of the belief that serum sickness could be correlated with the antigen-antibody relation with the body.

Tuft and Ramsdell (12–14) in a series of articles studied antibody formation subsequent to injection of horse serum. In the last one of this series they found that antibodies appeared both before and after the serum sickness or sometimes none appeared. They noted no regularity, and reached the conclusion that no clear relationship could be demonstrated between antibodies and the basic mechanism of serum sickness.

As the foregoing shows, the evidence regarding the relationship of antibody and antigen to the appearance of serum sickness in man is by no means uniform or definite. In our studies of the antigen and antibody curves in rabbits developing serum sickness, we find much the same irregularities which Tuft and Ramsdell report, and essentially no similarities to the results which Longcope and Rackemann and MacKenzie and Leake have noted. It does not appear possible to point out any relationship between the precipitin production, the disappearance of the antigen and the reaction of serum sickness in the rabbits we studied.

As discussed previously, the incubation period in serum sickness in rabbits is shorter than in man; it also appears that both the appearance of precipitins and disappearance of antigen take place more rapidly in rabbits than in man. These latter facts may account for the earlier appearance of serum sickness in rabbits; we do not imply that this is as cause and effect, but simply that it is possible that the immunological phenomena of appearance of precipitins and disappearance of antigens, like the incubation period for serum sickness, both occur more rapidly in rabbits. They might be considered as two parallel phenomena, which, however, do not regularly or constantly occur at the same rate in different individuals.

CONCLUSIONS

1. It has not been possible to demonstrate in rabbits affected with serum sickness any constant temporal relationship between precipitin and precipitinogen in the blood on one hand and of occurrence of serum sickness on the other hand.

2. It has not been possible to demonstrate any differences between the precipitin and precipitinogen curves in injected rabbits which develop serum sickness compared with injected rabbits which did not develop serum sickness.

3. There is therefore no evidence that we can directly associate the occurrence of serum sickness with the production or appearance of precipitins.

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THE TRANSFORMATION IN VITRO OF R PNEUMOCOCCI INTO S FORMS OF DIFFERENT SPECIFIC TYPES BY THE USE OF FILTERED PNEUMOCOCCUS EXTRACTS

By J. LIONEL ALLOWAY, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

Griffith in 1928 (1) demonstrated conclusively for the first time that pneumococci of one specific type may, by suitable methods, be converted into pneumococci of heterologous types. He effected the change by injecting subcutaneously into mice small amounts of culture of living, non-virulent R forms together with large amounts of heat-killed S pneumococci of a type other than that of the organisms from which the R cells were derived. Living virulent S organisms were recovered from heart's blood cultures of the animals. The type of the S organisms recovered was not that of the pneumococci from which the R organisms had been derived, but was that of the heat-killed S forms employed in the experiment. Hence a definite transformation in type had occurred. Rigid controls excluded the possibility of S forms being contained in the heat-killed suspensions.

Neufeld and Levinthal (2) and Dawson (3), using similar technic, confirmed Griffith's findings. All of these writers were unable to bring about a change of type in vitro. In a subsequent paper, however, Dawson and Sia (4) reported experiments in which it had been possible to effect a transformation of type in vitro. Their method was to inoculate small quantities of R pneumococci derived from S organisms of one type into blood broth containing anti-R serum and the heat-killed S pneumococci of another type. When solutions of S organisms broken up by freezing and thawing and subsequently heated to 60°C. were substituted for the whole bacteria, no transformation occurred.

In the studies just described, transformation of pneumococci of one specific type into those of another type was accomplished only by adding to living R cultures the whole heat-killed bodies of S pneumococci. In attempting to analyze the nature of this phenomenon it seemed desirable to determine whether the active principle responsible for the transformation could be extracted in soluble form from the S cells.

In the present paper experiments are reported on the use of cell-free,

heated and filtered extracts of S pneumococci in inducing conversion of R forms to the same specific type of Pneumococcus as that of the organisms from which the extract was prepared.

<div align="center">EXPERIMENTAL</div>

<div align="center">*Methods*</div>

Cultures—Cultures of R pneumococci used in the experiments were stock strains originally derived from type-specific S pneumococci by growth in broth containing 10 per cent homologous immune serum. The organisms from which the extracts were prepared were type-specific strains of S pneumococci the virulence of which had been maintained by frequent animal passages.

Preparation of Pneumococcus Extract.—Cultures of S pneumococci designed for extraction were grown in meat infusion broth, pH 7.8, containing dextrose 0.01 per cent. Just prior to inoculation the medium was boiled for 10 minutes, then cooled rapidly to a temperature suitable for growth. Cultures were grown under a seal of vaseline or liquid paraffin for 10 or 12 hours, depending on the density of growth.

Organisms from 5 l. of broth culture of S pneumococci so prepared were thrown down by centrifugation and were collected in a volume of approximately 40 cc. of sterile distilled water. The suspension was placed in a heavy pyrex tube and immediately covered with sterile liquid paraffin. The concentrated suspension was then frozen and thawed rapidly until the organisms were broken up, seven or eight freezings usually being necessary. Dry ice in alcohol was used to freeze the mixture. The preparation was immediately placed in a water bath and heated to 60°C. for 30 minutes, following which it was centrifuged at high speed for $2\frac{1}{4}$ hours. The supernatant fluid was diluted with sterile distilled water to a volume of 500 cc., made alkaline by the addition of 1.0 cc. of normal sodium hydroxide, and passed through a Berkefeld N filter. The filtrate was partially neutralized by the addition of 0.7 cc. of normal hydrochloric acid. Then, by vacuum distillation, at low temperature, the solution was concentrated to a volume of 50 cc., that is to 1/100 the volume of the original broth culture. The extract was finally heated in a water bath to 60°C. for 10 minutes to insure sterility. All extracts were tested for sterility by culture in broth and by animal inoculation. In no instance were viable pneumococci isolated from the heated filtrates.

Anti-R Serum.—The anti-R serum used originally was prepared by the intravenous injection into rabbits of suspensions of heat-killed R pneumococci. It was subsequently found that normal hog serum[1] served equally well. Since hog serum

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[1] Recent studies of Sia (5) have shown that normal hog serum has definite antagonistic properties against S forms of pneumococci. These findings have been confirmed by Kelley in this laboratory who found also (6) that the anti-R titre of normal hog serum is frequently as high as 1/512, the average being slightly lower.

was more easily obtained in large quantities it was substituted for anti-R rabbit serum.

Cultural Technic.—The *in vitro* experiments of Dawson and Sia'(4) showed that the size of the inoculum of the R cells exerts a determining influence on the result. Small inocula are essential for transformation. The present experiment was prepared as follows: One drop of an R culture prepared by diluting 0.1 cc. of an 8 hour culture in 5 cc. of sterile broth, was placed in a tube containing 1.5 cc. of broth, 1.0 cc. of the filtered extract, and 0.3 cc. of hog serum. The mixture was immediately covered with a thick layer of sterile liquid paraffin and incubated at 37°C. Transfers were made serially every 24 hours, 1 drop of culture being carried over to each tube of the second series. No experiment was considered negative until at least five subcultures had been made. As subcultures were prepared, transfers were also made to blood agar plates, so that the characteristics of the colonies might be studied. Whenever smooth colonies were noted, one such colony was picked and transferred to blood broth. The organisms from the cultures were subsequently typed and tested for virulence in mice.

Conversion of an R Strain Derived from Type II Pneumococci into a Type III S Strain

Since the filtered extracts were found to vary considerably in concentration and there was variation also in the behavior of cultures, even when grown in the same lot of extract, it was found advisable to set up several tubes for each experiment. The protocol and results of a characteristic experiment are given in Table I.

From Table I it can be seen that when an R strain of Pneumococcus derived from Type II S organisms was grown in broth containing a filtered extract of Type III S organisms and a small amount of anti-R serum, viable S pneumococci were recovered after 24 hours which were specifically agglutinable in Type III antiserum. They were encapsulated, formed large flat mucoid colonies on blood agar, and exhibited all the characteristics of Type III S pneumococci. Moreover, these organisms in the first culture after isolation were highly virulent for mice as is shown in Table II.

The acquisition of virulence seems rather remarkable in view of the fact that the R strain of Pneumococcus prior to modification was completely avirulent. Inocula of 0.75 cc. of the original R strain injected intraperitoneally into mice did not cause death, and on many occasions when the organisms from 15 cc. of culture were injected subcutaneously the animals lived. After transformation the newly

TABLE I

Conversion of Strain of R Pneumococcus Derived from Type II S into Type III S by Means of a Filtered Extract of Type III S Pneumococci

Tube	Amount of broth	Strain of R pneumococcus	Filtered extract of Type III S pneumococci	Anti-R serum (normal hog)	Type of colonies*	Specific agglutinability of S colonies
	cc.		cc.	cc.		
1	1.5	D 39 R**	1.0	0.3	R and S	Type III
2	1.5	D 39 R	1.0	0.3	R and S	Type III
3	1.5	D 39 R	1.0	0.3	R and S	Type III
4	1.5	D 39 R	1.0	0.3	R and S	Type III
5	1.5	D 39 R	—	0.3	R only	—
6	1.5	—	1.0	0.3	Sterile	—

* Determined by plating on blood agar after 24 hours growth.

** R strain derived from Type II S pneumococci by growth in homologous immune serum broth.

TABLE II

Virulence Test in Mice of Pneumococcus Type III Derived from an R Strain (D 39 R*) by Use of a Filtered Extract of Type III S Pneumococci

Mouse	Amount of culture**	Result
	cc.	
1	0.1	D in 24 hrs.
2	0.01	D in 26 hrs.
3	0.001	D in 24 hrs.
4	0.0001	D in 26 hrs.
5	0.00001	D in 60 hrs.
6	0.000001	D in 5 days
7	0.0000001†	S 7 days

* R strain derived from Type II S pneumococcus by growth in homologous immune serum broth.

** Type III S pneumococcus derived from D 39 R by means of filtered extract of Type III S.

† This inoculum seeded into agar yielded no growth.

D = died.

S = survived.

derived Type III culture proved fatal to mice in amounts as small as 0.000,001 cc.

Conversion of an R Strain Derived from Type II Pneumococci into a Type I S Strain

It seemed of interest, in view of the fact that an R strain derived from Type II organisms could be transformed through the use of an

TABLE III

Conversion of Strain of R Pneumococcus Derived from Type II S into Type I S by Means of a Filtered Extract of Type I S Pneumococci

Tube	Amount of broth	Strain of R pneumococcus	Filtered extract of Type I S pneumococci	Anti-R serum (normal hog)	Type of colonies	Specific agglutinability of S colonies
	cc.		cc.	cc.		
1 a	1.5	D 39 R*	1.0	0.3	R only	—
2 a	1.5	D 39 R	1.0	0.3	R only	—
3 a	1.5	D 39 R	1.0	0.3	R only	—
4 a	1.5	D 39 R	1.0	0.3	R only	—
5 a	1.5	D 39 R	—	0.3	R only	—
6 a	1.5	—	1.0	0.3	Sterile	—

Original culture (above)

First subculture

1 b	1.5	From Tube 1 a	1.0	0.3	R only	—
2 b	1.5	From Tube 2 a	1.0	0.3	R only	—
3 b	1.5	From Tube 3 a	1.0	0.3	R only	—
4 b	1.5	From Tube 4 a	1.0	0.3	R only	—
5 b	1.5	From Tube 5 a	—	0.3	R only	—

Second subculture

1 c	1.5	From Tube 1 b	1.0	0.3	R and S	Type I
2 c	1.5	From Tube 2 b	1.0	0.3	R and S	Type I
3 c	1.5	From Tube 3 b	1.0	0.3	R and S	Type I
4 c	1.5	From Tube 4 b	1.0	0.3	R and S	Type I
5 c	1.5	From Tube 5 b	—	0.3	R only	—

*D 39 R = an R strain derived from Type II S pneumococci by growth in homologous immune serum.

extract from Type III cells into typical Type III pneumococci, to determine whether these same R forms might be changed into Type I pneumococci by means of a Type I extract. Such an experiment is recorded in Table III. It will be noted that in this instance no trans-

formation in type occurred until the second subculture in the extract medium had been made.

From Table III it can be seen that after an R strain of Pneumococcus derived from Type II S pneumococci was passed through three transfers in broth containing anti-R serum and a filtered extract of Type I culture, organisms were recovered which showed the characteristics of Type I pneumococci. They formed smooth colonies on blood agar, agglutinated specifically in Type I antiserum, possessed capsules, and were virulent for mice.

The conversion of R forms into organisms of Type I has been consistently more difficult than their transformation into Type III pneumococci. In the former instance three, four, and even five subcultures in the extract medium have been necessary.

DISCUSSION

The conditions affecting the activity of the filtered extracts are on the whole similar to those influencing the potency of the bacterial suspensions used by Griffith and by Dawson in their experiments. Autolysis of cells during the preparation of the extract must be minimized to avoid loss of activity. Aging of the extract tends to diminish its potency. Growth under anaerobic conditions facilitates but is not essential for the transformation.

The nature of the rôle of anti-R serum in the conversion is not fully understood. It is known (7) that growth of R cultures in broth containing anti-R serum usually results in their reversion to S organisms of the specific type from which they were originally derived. It has been found that transformations occur when normal sheep, rabbit, guinea pig, horse, or human serum is employed. These sera differ markedly in their anti-R titre and the observations so far seem to indicate that some other property of these sera may be the one concerned in the reaction. Thus far it has not been possible entirely to dispense with serum in the culture medium.

Despite the fact that the capsular polysaccharide of the Pneumococcus determines its type specificity, this substance alone, when added in chemically purified form, has not been found effective in causing transformation of R organisms derived from pneumococci of one type into S forms of other types. Numerous experiments in

which specific polysaccharides were used in varying dilutions together with anti-R serum and R cultures yielded only negative results.

It is believed that the procedures carried out in the preparation of the filtered and heated extracts exclude the possibility of any viable pneumococci being carried over in the extracts. It seems very unlikely, despite recent reports on filtrable forms of bacteria, that any living element can persist after the heating and filtration to which the material is subjected. Careful controls of sterility, including frequent serial transfers and mouse inoculation, have been invariably negative. Consequently the appearance in R cultures grown in the extract medium, of S forms belonging to the same specific type as that of the extracted pneumococci, is believed to indicate that the R cells originally derived from one type of Pneumococcus have acquired the characteristics of S forms of a different type. Once the organisms have assumed the specific characters, they remain true to form through repeated transfers in ordinary media and through repeated animal passages.

Filtered extracts prepared as described are not invariably active. Several preparations have failed to effect a change despite repeated sub-cultures in the extract-containing medium. Moreover, out of a group of six tubes identically prepared, so far as can be determined, two or three may give positive results while the others remain negative. Although attempts to convert R pneumococci derived from Type I or Type III strains into S organisms of other types through the use of extracts have thus far been unsuccessful, it seems likely that by proper modification of the technic, these changes may be accomplished. Marked differences are encountered in the ease with which R organisms may be transformed even when suspensions of heat-killed cells are used. In view of the additional manipulation incident to the preparation of extracts, and the probable loss of the specific principle responsible for the change, it is not surprising that failures are encountered when such extracts are employed.

When R pneumococci change into the S forms they always acquire the property of producing the specific capsular substance. The immunological specificity of the encapsulated cell depends upon the chemical constitution of the particular polysaccharide in the capsule. The synthesis of this specific polysaccharide is a function peculiar to

S forms of pneumococci. However, since the R cells under suitable conditions have been found to develop again the capacity of elaborating this specific material, it appears that in them this function is potentially present, but that it remains latent until activated by special environmental conditions. The fact that an R strain derived from one type of Pneumococcus, under the conditions defined in this paper, may be caused to acquire the specific characters of the S form of a type other than that from which it was originally derived implies that the activating stimulus is of a specific nature.

Since this paper was submitted for publication, two articles by Dawson and Sia (8, 9) have appeared on the transformation of pneumococcal types *in vitro*. In the second of the two papers the authors describe experiments in which they were unable to effect a transformation of type *in vitro* by using extracts made under anaerobic conditions by freezing and thawing 20 or more times suspensions of type-specific pneumococci. In a personal communication, Dawson states he has found recently that suspensions of S pneumococci which are frozen and thawed less than eight times are still active in inducing transformation in type.

CONCLUSIONS

1. Avirulent R pneumococci derived from S forms of a specific type may be changed by growth in broth containing anti-R serum and a heated, filtered extract of S pneumococci of a different type, into virulent S organisms identical in type with the bacteria extracted. This has been accomplished in the case of R strains derived from Type II pneumococci, employing extracts prepared from Type III and Type I S forms.

2. The constituents of the extract supply an activating stimulus of a specific nature in that the R pneumococci acquire the capacity of elaborating the capsular material peculiar to the organisms extracted.

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THE ACCUMULATION OF IRON IN TUBERCULOUS AREAS

II. Survival Time of Tuberculous Rabbits Injected with Ferric Chloride

BY VALY MENKIN, M.D.

(From the Department of Pathology, Harvard University Medical School, Boston)

Previous studies have demonstrated that certain foreign substances injected into tuberculous animals accumulate in higher concentrations in tubercles than in normal tissues.

Bowman, Winternitz, and Evans by microscopic studies (1) found that, in experimental tuberculosis, trypan blue injected intravenously stained tubercles in the liver. They pointed out the great affinity of giant and epithelioid cells for this vital dye which was always found as granules within these cells. Lewis (2) showed that if the cornea of a rabbit is inoculated with a living culture of the tubercle bacillus, a progressive lesion results characterized by an intense congestion of the conjunctiva. If the animal is given an intravenous injection of trypan red 24 hours or more after such inoculation, the fluid in the anterior chamber of the inoculated eye always becomes colored. Wells and Hedenburg (3) have demonstrated that iodide of potassium injected subcutaneouly enters freely into tubercles. Furthermore the proportion of iodine in such tubercles is greater in the caseous areas than in the cellular peripheries of the tubercles. This property is believed by them to depend on no specific character or affinity of the tubercle itself, for other necrotic tissues also take up more iodine than do normal tissues.

In previous communications (4, 5) it has been shown that a vital dye, trypan blue, or iron in the form of its salt, ferric chloride, when injected into the blood stream rapidly accumulates in an area of inflammation, where the substance is fixed and fails to drain to the tributary lymph nodes. Subsequent experiments (6, 7) demonstrated that repeated daily intravenous injections of ferric chloride in rabbits were followed by an accumulation of iron in tuberculous areas of lungs. The iron was shown to accumulate in the caseous areas of tubercles. The above experiments suggest that the accumulation of

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iron in tuberculous areas may alter the character or course of development of the disease.

Bovine tuberculosis was induced in weighed rabbits by the intravenous injection of 0.001 mg. of a saline suspension of Ravenel strain. 26 days later the animals were divided into two groups. One group was kept to serve as control. Intravenous injections of 0.25 per cent ferric chloride were started in the other group as follows: 1 cc. on the 1st day; 2 cc. on each of the 2 following consecutive days; 4 or 5 cc. on each of the next 6 days; and 6 cc. on almost each of the successive days for a period of about 2 weeks. Subsequently, injections of the ferric salt (6 cc. each time) were made every other day for a period of 6 weeks. The ferric salt solution was kept on ice when not in use. It was gently heated before each intravenous injection. The latter were always given very slowly.

Both groups of animals were weighed every week during the course of the iron injections The weighing was always performed at the same time of day. The animals were so distributed that usually two or three rabbits were kept in a cage. As a rule a control tuberculous animal was kept in the same cage with an experimental animal in order to standardize environmental conditions as closely as possible. When a rabbit died an autopsy was performed. Usually specimens of lung, liver, spleen, and kidney were fixed in 10 per cent formalin and stained with hematoxylin and eosin for microscopic examination. Only one animal (Rabbit 2-07) showed intense coccidial infection in the liver superimposed on its induced tuberculosis. Control Rabbit 2-14 revealed an unusually large caseating tuberculous focus in the marginal ear vein with pronounced thrombosis of the vessel. This area represented the original site of inoculation with tubercle bacilli.

The total amount of ferric chloride injected in each rabbit varied from 49 to 238 cc. of a 0.25 per cent solution. It was found in the course of this investigation that ferric chloride in the concentrations used is toxic for some rabbits. Injection in these animals may be followed by immediate death. Slight warming of the ferric chloride solution and slow injection failed to prevent such accidental fatalities. It was found, however, that small initial doses of the ferric salt evidently increase the tolerance of the animal, so that subsequently, considerably higher amounts can usually be injected intravenously without any untoward effect.

Rabbits which developed signs of upper respiratory infection were discarded. Two rabbits died 3 weeks after inoculation with tubercle bacilli. The gross appearance of the lungs showed definitely that death was not due to tuberculosis but rather to a pneumonic process. These two rabbits were discarded from the series. One animal with an upper respiratory infection which had received two small doses of ferric chloride died immediately after the third intravenous injection. It appears as if animals with superimposed infection tolerate very poorly intravenous injections of the ferric salt.

Rabbits 1-50, 5-51, 5-55, 1-46, 5-96, and 5-14 were each injected intravenously

with 0.001 mg. of bovine Ravenel strain of tubercle bacilli. These six animals were not injected at the same time as the remaining ten rabbits of the series. Rabbits 1-50, 5-51, and 5-55 served as controls to Rabbits 1-46, 5-96, and 5-14. Daily injections of the ferric salt were begun at a later stage of the disease *i.e.* 1 month to 40 days after inoculation with tubercle bacilli; and these injections were kept up for a shorter interval than in the remaining rabbits of the series. Periodic weekly weights were not recorded in the six forementioned rabbits. Furthermore, the weights of Rabbits 5-55, 5-96, and 5-14 as shown in Table I were obtained several weeks before inoculation with tubercule bacilli.

The results of all the experiments may for convenience be listed in three separate groups as follows: (1) Correlation in both control and experimental animals of changes in weight with time. (2) Comparison of the survival time in both groups. (3) Pathological appearance of some of the organs, notably the lungs in both control and experimental rabbits at the time of death.

1. The weight changes in the control and experimental animals are plotted on Chart 1. The individual weights at the outset of the experiments showed definite variations, but it is clear that these initial differences were more or less evenly distributed between control and experimental groups. The control group showed, in all cases but one, an initial increase in weight followed by a sharp decline terminating in death. With the exception of Rabbit 2-14 (V[1]) the decline in weight of each control rabbit began about 4 weeks after inoculation with tubercle bacilli at the time when injections of ferric chloride were started in the experimental group. In the latter (excepting Rabbit 2-07 (VII)) a steady increase in weight continued during part of the period of ferric chloride injections. At a time when most of the control animals were already dead the experimental rabbits for the most part were attaining a maximum weight far above that which had been reached by any of the controls. Toward the end of the period of ferric chloride injections, a decline in weight began. This fall became abrupt after the injections of iron ceased and terminated in the death of the rabbit.

2. A comparison of the results in the survival time of control and experimental rabbits is shown in Table I. It is clear that if Rabbit 2-14 in the control group and Rabbit 2-07 in the experimental group

[1]Roman numerals refer to the numbers on Chart 1.

3. A study of the morphological appearances, in the gross and microscopically, of the lungs in particular and of some of the other organs failed to reveal any striking differences between the control and the experimental animals. Since both groups of animals were allowed to die with generalized caseating tuberculosis, and since the organs were compared only at death, the observations do not in any way rule out a possible effect of ferric chloride on the rate of development of the tuberculous lesion. Experiments are now under way upon this aspect of the problem. Postmortem evidences in the present series of animals show that typical tuberculous lesions have developed both in experimental and control rabbits. There is no definite indication at autopsy that the tuberculous lesion is modified by the repeated injections of ferric chloride.

A brief summary of a few typical protocols of the postmortem examination (of the lungs in particular) is presented.

Rabbit 1-50.—Control; survival time 69 days; lungs show massive confluent caseating tuberculosis with several cavities; microscopic examination reveals several caseating areas with calcium deposits; diffuse mononuclear infiltration throughout lung parenchyma; no gross evidence of tuberculosis in liver and spleen; a few discrete foci in the kidneys.

Rabbit 2-04.—Control; survival time 79 days; lungs moderately enlarged; large confluent caseating tubercles at base with considerable adherence to parietal pleura. Numerous discrete foci at apex; two cavities in lower lobe. Microscopic section reveals many caseous foci with typical epithelioid and mononuclear phagocytic cells at periphery; occasional invasion of bronchiole wall by tubercle. Few small tuberculous foci in spleen. Many caseating tubercles in left kidney.

Rabbit 2-06.—Control; survival time 52 days; lungs show many patches of large caseous foci; small discrete tubercles in the remaining parts. Microscopic section characterized by numerous small caseous foci some of which are adjacent to bronchi and have penetrated latter. On another section large tuberculous foci with coarse deposits of calcium in areas of caseation. Both kidneys and spleen show many discrete tubercles.

Rabbit 2-13.—Experimental; a total amount of 238 cc. of 0.25 per cent of ferric chloride injected; survival time 130 days; lungs definitely enlarged and congested; extensive confluent caseating tuberculosis at base; a large pus cavity at base of left lung; apices involved with caseating foci. Microscopic examination reveals extensive engorgement of capillaries at periphery of tubercles; numerous confluent caseous areas containing large calcium deposits; diffuse mononuclear phagocytic cell infiltration in parenchyma surrounding caseous areas; slight fibroblastic reaction around tubercles. Hemosiderin-like granules in spleen, liver, and to a slight extent in kidney.

Rabbit 2-85.—Experimental; a total amount of 226 cc. of 0.25 per cent ferric chloride injected; survival time 128 days; lungs distinctly enlarged and congested; left lung more involved than right one; shows extensive confluent caseating tubercles; within some areas of caseation spots of brown pigmentation seen. Right lung shows confluent tuberculosis at base and apex; cavity formation at base; discrete foci elsewhere. Microscopic examination reveals larger number of caseous areas with capillary congestion at the peripheries and with only very slight connective tissue proliferation. Some caseous areas contain large calcium deposits; typical mononuclear phagocytic and epithelioid cells at the peripheries of caseous areas; diffuse lymphoid infiltration elsewhere; hemosiderin-like granules seen in spleen, liver, and to a slight extent in kidney.

Rabbit 1-46.—Experimental; a total amount of 49 cc. of 0.25 per cent ferric chloride injected; survival time 88 days; lungs riddled with extensive caseous foci some of which are confluent; cavity formation at apices. Liver black in appearance; spleen and kidneys reveal many caseating tubercles. Microscopic examination of lungs shows numerous large caseous tubercles many of which contain calcium deposits; extensive congestion and engorgement of vessels in some fields; some bronchi plugged with large amount of cellular exudate composed chiefly of polymorphonuclear and mononuclear phagocytic cells. Liver cells loaded with fine brown hemosiderin-like granules; Kupffer cells also filled with similar deposits but coarser in appearance.

DISCUSSION

The foregoing series of experiments demonstrates that repeated intravenous injections of ferric chloride are followed by a marked increase in the survival time of tuberculous rabbits. During part of the period of these injections, animals show definite increases in weight exceeding any weights attained in the control group. However both control and experimental animals die of generalized tuberculosis with typical lesions which at the time of death are practically indistinguishable from each other. It has been shown in previous communications (6, 7) that such repeated intravenous injections of ferric chloride are followed by an accumulation of iron in the caseous areas of the lung. Whether or not the entire mechanism involved in prolonging the life of tuberculous rabbits is linked up with this accumulation of iron in the tubercles remains to be seen. Experiments are now under way to answer this question. Furthermore it is conceivable that by varying the infecting dose of tubercle bacilli, the concentration of ferric chloride, or the number of injections, even more striking results might be obtained. Such experiments are also being conducted and will be reported in a future communication. Walbum (8) as a result of a

study of tuberculous mice and guinea pigs reports that iron amongst many other metals has no effect on the disease, or at most one to delay it slightly, the animals dying of typical tuberculosis.   This investigator injected his animals repeatedly with an iron salt, either by the subcutaneous or the intraperitoneal route.   In previous studies on inflammation (4, 5, 9) it has been shown by the writer that foreign substances as *e.g.* dyes, iron, proteins, or bacteria injected into an area of subcutaneous or peritoneal inflammation are fixed *in situ* and fail to disseminate readily into the regional lymphatics.   In view of this it is quite likely that repeated subcutaneous or intraperitoneal injections of iron, which acts as an inflammatory irritant at the point of its injection, may fail to produce the same results elsewhere in the body as those obtained by the intravenous route.

CONCLUSIONS

Repeated intravenous injections of ferric chloride are followed by an increase in the survival time of tuberculous rabbits.   In the particular series of experiments reported this increase amounts to about 78 per cent over the average survival time of control rabbits.

Tuberculous animals repeatedly injected with ferric chloride increase in weight during part of the period of these injections.   The level reached in the series studied markedly exceeds that attained by control rabbits.

Both control and experimental animals die of generalized tuberculosis.   There is no indication at the time of death of any differences in the degree of pathological involvement between the two groups of animals.

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THE EFFECT OF BILATERAL SUPRARENALECTOMY IN ADULT ALBINO RATS ON THE NATURAL AND ACQUIRED RESISTANCE TO BARTONELLA MURIS ANEMIA

BY J. MARMORSTON-GOTTESMAN, M.D., AND DAVID PERLA, M.D.

(From the Laboratory Division, Montefiore Hospital, New York)

By work in this laboratory and that of other investigators, it has been shown that the suprarenal glands are essential in the mechanism of natural resistance (1–12). In the case of suprarenalectomized rats, this resistance can be raised almost to normal by injection of the hormone of the suprarenal cortex (13–15). In the present communication further data are presented on the effect of bilateral suprarenalectomy in the rat on natural and acquired resistance to *Bartonella muris* anemia.

The Effect of Bilateral Suprarenalectomy on the Acquired Resistance to Bartonella muris Anemia

The rat is spontaneously infected with *Bartonella muris* between the 4th and 5th week of life (16). It becomes a carrier and acquires immunity to the infection (17). The spontaneous recurrence of infection following splenectomy with the development of anemia in the adult rat of carrier stock is indicative of a depression in the acquired resistance to *Bartonella muris*.[1]

The rats used in this experiment were bred and raised in our laboratory and were carriers of *Bartonella muris*. Ten albino rats of approximately 3 months of age were suprarenalectomized. On the 6th day after suprarenalectomy each of these was injected intraperitoneally with 2 cc. of whole blood of anemic adult splenectomized rats, drawn at the height of infection. The red cell count and the percentage of hemoglobin were determined and blood smears examined daily.

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[1] For a review of the literature on *Bartonella muris* anemia the reader is referred to Marmorston-Gottesman and Perla (17) and to Reitano (18).

In some instances a few *Bartonella* bodies were found in the red cells during 1 or 2 days following the injection of the infected material but no anemia developed in any of the rats. Ford and Eliot (19) have shown that 0.0001 cc. of blood of an anemic rat is sufficient to transmit the anemia to splenectomized rats of non-carrier stock. The suprarenalectomized rats of carrier stock, therefore, withstand a quantity of infecting material 20,000 times as great as is sufficient to produce an anemia in the splenectomized rat of non-carrier stock. The acquired resistance of rats of carrier stock to *Bartonella muris* conferred by a first infection in early life, though strikingly depressed by splenectomy, is uninfluenced by suprarenalectomy.

The Effect of Bilateral Suprarenalectomy on the Natural Resistance of Adult Albino Rats to Bartonella muris Anemia

To determine the effect of suprarenalectomy on the natural resistance to *Bartonella muris* it was necessary to study this infection in rats of non-carrier stock. Rats of the Wistar strain are not carriers of *Bartonella muris* (19). Splenectomy in these rats is not followed by *Bartonella muris* anemia but the anemia will develop if they are subsequently exposed to rats of carrier stock for a few days.

The rats used in the experiments on natural resistance were bought directly from the Wistar Institute and were isolated. Preliminary tests indicated that these rats were not carriers of *Bartonella muris*. Ten suprarenalectomized 3 month old rats and one splenectomized rat of the same age and stock were each injected intraperitoneally on the 6th day following the operation with 1 cc. of whole blood of anemic adult splenectomized rats, drawn at the height of the anemia. Four male and two female normal rats of the same age and stock were each injected intraperitoneally with the same quantity of blood of an anemic rat. The red blood cell count and the percentage of hemoglobin were determined and blood smears were examined daily.

From Table I it is seen that eight of the suprarenalectomized rats died within a period of 3 days following the injection of infecting material and two on the 5th and 6th day. In most instances the rats died before sufficient time had elapsed for the development of a severe anemia. Death was apparently caused by the toxemia of the infection. Rats surviving over a period longer than 2 days after injection developed an anemia. The histological changes character-

istic of acute *Bartonella muris* anemia (17) were found even in those instances in which examination of the blood had failed to reveal the presence of an anemia. Focal necroses in the liver and spleen and erythrophagocytosis by endothelial cells of the spleen with exhaustion destruction of the pulp were present. The splenectomized rat survived.

The control unoperated Wistar rats injected with the same amount of infecting material developed a severe anemia. All of these recovered. The red cell count and the percentage of hemoglobin began to drop within 5 days and rapidly reached a level below 2 million red cells per cubic millimeter and 10 per cent hemoglobin. The females developed an anemia less severe and more transient than the males. This sex variation in the severity of the anemia has been repeatedly observed in our studies on splenectomized rats of carrier stock.

These observations are at variance with those of Ford and Eliot who were unable to transmit the anemia to normal rats of non-carrier stock. Adler (20, 21) succeeded in infecting normal mice by repeated passage of *Bartonella muris ratti*. After several transfers the virulence of the organism was enhanced so that the virus produced an anemia in mice. Mice that had recovered from a previously induced infection could not be reinfected with this strain.

The natural resistance to *Bartonella muris* infection is lowered by suprarenalectomy. The rapidity with which the anemia developed was no greater nor was the anemia more severe in the suprarenalectomized rats than in the normal rats, but all the suprarenalectomized rats died of the toxemia. The mortality from suprarenalectomy alone in rats during the first 3 weeks is less than 10 per cent in our laboratory. The mortality from *Bartonella muris* anemia in splenectomized rats of carrier stock is 30 per cent.[2]

In a second experiment on the effect of suprarenalectomy on the natural resistance of rats to *Bartonella muris* the effect of the injec-

[2] Adler (21) has pointed out that there are variations in virulence in different strains of *Bartonella muris*. The mortality of splenectomized rats in Jerusalem is 96 per cent, in Hamburg it is 30 per cent and in Sumatra it is 70 per cent (Kurschner and Timmerman (22)). The high mortality in these rats may be due to climatic conditions and not to any marked variation in the potency of a strain. We have noted a higher mortality rate in summer than in winter.

TABLE I

The Effect of Bilateral Suprarenalectomy on the Natural Resistance of Adult Albino Rats to B. muris (B. muris-Free Stock Used in the Experiment). Effect of Injections of 1 Cc. of Whole Blood of Anemic Rat*

Rat No.	Sex	Operation	Amount of blood injected (cc.)	Days after operation	Red blood cell count and hemoglobin — No. of days after injection								Remarks
					1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	
1953	M	Bilat. Suprar.	1	7	9,500 T 110%	8,000 T 90%	7,500 T 90%	9,500 T 85%	Died				
1954	M	"	1	7	9,300 T 105%	6,000 T 95%	7,500 T 85%	Died					
1955	M	"	1	7	9,300 T 110%	9,500 T 110%	Died						
1956	M	"	1	7	10,200 T 110%	5,900 T 60%	6,100 T 85%	"					
1957	M	"	1	7	10,100 T 110%	8,300 T 100%	9,500 T 110%	"					
2041	M	"	1	7	10,200 T 110%	Died							
2042	M	"	1	7	10,200 T 115%	9,700 T 100%	7,400 T 90%	"					
2043	M	"	1	7	10,000 T 118%	Died							

No.	Sex												
2044	M	Bilat. Suprar.	1		11,000 T 118%	Died							
2045	M	"	1	7	9,750 T 120%	"							
2040	M	Spleen	1	7	10,100 T 105%	8,500 T 105%	9,500 T 110%	4,100 T 60%	2,200 T 35%	2,000 T 10%	4,000 T 20%	Recovered	
2039	M	"	0	7	10,000 T 115%	9,800 T 110%	10,500 T 115%	10,500 T 103%	9,800 T 100%	9,500 T 105%	"		
2048	M	Control	1		11,000 T 110%	10,200 T 100%	6,500 T 60%	2,000 T 40%	2,200 T 30%	1,800 T 35%	4,000 T 60%	"	
2049	M	"	1		11,000 T 90%	9,700 T 110%	4,500 T 65%	3,000 T 45%	2,400 T 40%	3,000 T 32%	4,500 T 65%	"	
2106	M	"	1		11,500 T 110%	11,400 T 80%	6,900 T 80%	7,000 T 80%	6,500 T 70%	3,800 T 58%	5,500 T 70%	7,000 T 90%	Recovered
2107	M	"	1		10,000 T 105%	10,500 T 105%	7,500 T 90%	5,500 T 78%	6,000 T 50%	5,600 T 70%	6,000 T 80%	8,500 T 100%	"
2110	F	"	1		9,500 T 110%	9,000 T 90%	7,000 T 70%	5,500 T 58%	5,200 T 55%	6,500 T 80%	6,500 T 95%	9,000 T 110%	"
2111	F	"	1		10,500 T 105%	9,500 T 100%	6,000 T 80%	5,000 T 45%	4,500 T 40%	4,900 T 80%	6,500 T 95%	7,500 T 110%	"

* Blood obtained from splenectomized rat at height of anemia.

The letter "T" is used in place of the last three zeros in the red cell count. The hemoglobin is expressed in percentages as calculated from readings with the Dare hemoglobinometer.

TABLE I—*Concluded*

Rat No.	Interval between injection of *B.muris* and death	Pathology
	days	
1953	5	Typical changes characteristic of *B. muris* anemia
1954	4	" " " " " "
1955	3	" " " " " "
1956	4	" " " " " "
1957	4	" " " " " "
2041	1	No pathological change characteristic of *B. muris* anemia
2042	4	Typical changes characteristic of *B. muris* anemia
2043	1	Congestion of pulp of spleen with enlarged follicles and erythrophagocytosis but no other changes characteristic of *B. muris* anemia
2044	1	No pathological change characteristic of *B. muris* anemia
2045	1	" " " "

TABLE II

The Effect of Bilateral Suprarenalectomy on the Natural Resistance of Adult Albino Rats to B. muris (B. muris-Free Stock Used in the Experiment). Effect of Injections of 0.2 Cc. of Whole Blood of Anemic Rat*

Rat No.	Sex	Operation	Days after operation	Red blood cell count and hemoglobin									
				No. of days after injection									
				1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day
2100	M	Bilat. Suprar.	7	8,500 T 105%	8,900 T 95%	8,250 T 100%	10,000 T 90%	8,000 T 80%	8,000 T 70%	6,000 T 80%	90%	7,000 T 90%	8,000 T 95%
2101	M	"	7	11,000 T 110%	10,000 T 110%	6,200 T 95%	8,000 T 90%	7,200 T 82%	7,600 T 63%	6,200 T 80%		6,800 T 85%	8,500 T 100%
2102	M	"	7	10,000 T 110%	11,500 T 85%	9,000 T 90%	8,000 T 100%	8,600 T 89%	7,900 T 75%	8,600 T 80%	80%	6,800 T 85%	10,500 T 100%
2103	M	"	7	9,150 T 110%	11,000 T 110%	10,000 T 100%	8,200 T 90%	8,200 T 95%	8,800 T 85%	7,500 T 60%	5,200 T 70%	7,400 T 85%	3,000 T 90%
2104	M	"	7	10,000 T 110%	8,500 T 110%	9,000 T 100%	8,900 T 90%	6,200 T 63%	3,000 T 70%	7,500 T 80%	9,000 T 82%	9,000 T 85%	9,500 T 90%
2105	M	"	7	10,500 T 105%	9,000 T 95%	8,000 T 95%	8,500 T 80%	3,000 T 80%	7,800 T 70%	9,000 T 80%	9,000 T 100%	8,500 T 110%	
2108	M	Normal		10,500 T 110%	8,800 T 105%	8,750 T 95%	6,500 T 75%	6,000 T 60%	6,900 T 70%	7,000 T 95%	8,500 T 100%	9,000 T 110%	
2109	M	"		9,800 T 110%	9,500 T 100%	7,000 T 70%	6,500 T 70%	6,400 T 75%	7,500 T 100%	7,500 T 100%	9,000 T 110%	9,000 T 110%	

* Blood obtained from splenectomized rat at height of anemia. The letter "T" is used in place of the last three zeros in the red cell count. The hemoglobin is expressed in percentages as calculated from readings with the Dare hemoglobinometer.

infecting material are used. The normal adult rat of Wistar stock possesses a relatively high natural resistance to spontaneous infection with this organism.

Bilateral suprarenalectomy in Wistar rats lowers the natural resistance to a subsequent infection with *Bartonella muris*. This procedure does not alter the type of tissue response to the virus but lowers the natural resistance of the rat to toxic effects of the infection. The acquired immunity to *Bartonella muris* conferred by a first infection is not broken down by subsequent suprarenalectomy.

The mechanisms of acquired and natural resistance are dependent on different physiological processes in the organism and are not merely quantitative variations of the same process as is generally assumed.

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CONDITIONS INFLUENCING THE DISAPPEARANCE OF LIVING BACTERIA FROM THE BLOOD STREAM

By PAUL R. CANNON, M.D., F. L. SULLIVAN, AND E. F. NECKERMANN

(From the Department of Pathology and the Otho S. A. Sprague Memorial Institute, The University of Chicago, Chicago)

PLATES 5 AND 6

It is generally agreed that bacteria introduced intravenously into animals are quickly removed from the blood stream and localized or "fixed" in the phagocytic cells, especially those of the reticulo-endothelial system. The primary removal apparently follows more or less closely the conditions governing the disposal of particulate material under similar circumstances. The rate of disappearance and the ultimate fate depend upon the number and type of microorganisms injected, their pathogenicity for the animal, the functional state of the phagocytes and, indirectly, of the body as a whole.

Various investigators have determined some of the conditions, *e.g.*, hunger, food ingestion, disease, principal sites of localization, which affect or modify the removal of bacteria from the blood stream in man and normal laboratory animals (1). But, aside from the few preliminary experiments of Hopkins and Parker (2) and Parker and Franke (3), practically no work has been reported concerning the effects of specific active immunization upon the removal of bacteria from the circulating blood. We wish to report the results of studies of this sort, emphasizing particularly certain morphological aspects of the problem.

Materials and Methods

Adult rabbits, usually males, were immunized by the intravenous injection of a 24 hour growth on agar slants of microorganisms suspended in 0.85 per cent solution of sodium chloride and heated at 60°C. for 1 hour. One series of animals was immunized against *Staphylococcus albus* and another series against *Bact. paratyphosum* B. As a rule, a total of from 4 to 6 cc. of the suspension was given in daily injections of 1 cc. each. After a period of time varying usually from 1

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to 3 weeks, these rabbits and normal ones of approximately the same size were simultaneously given intravenous injections of equal quantities of a 24 hour broth culture of the homologous living bacteria in order to determine the comparative rates of disappearance from the blood stream. Broth cultures were used so that the bacteria might be in a good state of dispersion with as few clumps as possible.

At intervals of 5, 15, 30, 45, 60 minutes, etc., blood was taken with aseptic precautions from the heart of the normal and the immune animal and plated in duplicate amounts in infusion agar, pH 7.3 to 7.5., the plates incubated at 37°C. and colony counts made at the end of 48 hours. Dilutions were required only in the sample of blood withdrawn 5 minutes after the intravenous injection. Such a method is subject to the usual error of interpretation that each colony does not necessarily represent one bacterium; therefore the results are recorded as the number of colonies per cubic centimeter of blood.

Smears and sections of the various organs and tissues were also made at different intervals in order to observe morphological changes as well as variations in concentration of the bacteria in the tissues of the normal and immunized animals For sections, the tissues were fixed in Zenker-formol solution, embedded in celloidin, sectioned at 10 microns and stained with hematoxylin-eosin-azur 11 and by the Claudius modification of the Gram stain (4).

We used a constant dosage of bacteria rather than a definite quantity per kilo of body weight because of the probability that much of the variation in weight may be due to unequal quantities of food in the gastrointestinal tract, different amounts of adipose tissue and differences in size of bone. For example, we found that in eight pairs of rabbits in which the initial differences in weight varied from 10 to 30 gm., the corrected weights after removal of the gastrointestinal tract ranged from 40 to 225 gm. There is necessarily an element of error no matter which procedure is followed but we believe that the injection of a constant dosage insures a more comparable method. In most of our experiments we have also determined the weights after the removal of the gastrointestinal tracts and have thus been able to analyze the results more satisfactorily.

Numerous experiments on relatively large numbers of animals are advisable in order to rule out individual variations in reactivity of "normal" animals. It is doubtful whether rabbits that have been infected with coccidia or have had snuffles or infected wounds are really normal in so far as the reactivity of macrophages is concerned. Furthermore, differences in nervous states, especially of the autonomic nervous system, as emphasized particularly by Müller (5) and Petersen (6), necessitate recognition of the importance of uniformity of handling, feeding and routine care so that reactivity may be as constant as is experimentally possible. In later stages of our work much less variation occurred, owing to the use of rabbits reared under exceptionally favorable conditions, and known to be absolutely free from "snuffles" and practically free from coccidial infection.

The Passage of Staphylococci through the Lungs of Normal and Immune Rabbits

Inasmuch as opinions vary concerning the relative importance of the lungs in the removal of bacteria from the blood stream, it is desirable to know just how and in what numbers microorganisms pass through this area. To determine this, equal amounts of a suspension of *Staphylococcus albus*, usually from 1 to 3 cc. of a 24 hour broth culture, were injected simultaneously into the marginal ear veins of normal and of actively immunized rabbits. At exactly 2 minutes, 4 minutes, 5 minutes and at later intervals up to 1 hour, the lungs were fixed in Zenker-formol solution, sectioned and stained.

Fig. 1 illustrates the findings in a normal rabbit 2 minutes after intravenous injection. This is a camera lucida drawing of two pulmonary veins and shows the staphylococci evenly dispersed in the lumina with occasional ones engulfed by leucocytes. The bacteria are compact and well stained, show no evidence of any agglutinative tendency and are apparently passing rapidly and extracellularly through the lungs on their way to the general systemic circulation. In the immunized animal at this period there was no increased number of bacteria within the pulmonary tissues, and although polymorphonuclear leucocytes were present in greater numbers than normal, practically none contained staphylococci. In neither rabbit were mononuclear cells particularly active in phagocytosis of the microorganisms. Sections taken from the lungs of normal and immune rabbits 4 and 5 minutes and later after injection showed practically no extracellular microorganisms and only a few in polymorphonuclear leucocytes and macrophages.

It is evident from these experiments that the countless numbers of staphylococci in the venous blood stream pass almost instantaneously through the blood vessels of the lungs of both normal and immune rabbits. A comparatively small number are caught within the capillaries; this may be due to mechanical obstruction or chance collisions with leucocytes. The bacteria show no evidences of agglutination or swelling such as will be seen in the livers and spleens of these same rabbits. We conclude that under the conditions of our experiments the lungs play an insignificant part in "fixing" such antigen as staphylococci.

to 3 weeks, these rabbits and normal ones of approximately the same size were simultaneously given intravenous injections of equal quantities of a 24 hour broth culture of the homologous living bacteria in order to determine the comparative rates of disappearance from the blood stream. Broth cultures were used so that the bacteria might be in a good state of dispersion with as few clumps as possible.

At intervals of 5, 15, 30, 45, 60 minutes, etc., blood was taken with aseptic precautions from the heart of the normal and the immune animal and plated in duplicate amounts in infusion agar, pH 7.3 to 7.5., the plates incubated at 37°C. and colony counts made at the end of 48 hours. Dilutions were required only in the sample of blood withdrawn 5 minutes after the intravenous injection. Such a method is subject to the usual error of interpretation that each colony does not necessarily represent one bacterium; therefore the results are recorded as the number of colonies per cubic centimeter of blood.

Smears and sections of the various organs and tissues were also made at different intervals in order to observe morphological changes as well as variations in concentration of the bacteria in the tissues of the normal and immunized animals For sections, the tissues were fixed in Zenker-formol solution, embedded in celloidin, sectioned at 10 microns and stained with hematoxylin-eosin-azur 11 and by the Claudius modification of the Gram stain (4).

We used a constant dosage of bacteria rather than a definite quantity per kilo of body weight because of the probability that much of the variation in weight may be due to unequal quantities of food in the gastrointestinal tract, different amounts of adipose tissue and differences in size of bone. For example, we found that in eight pairs of rabbits in which the initial differences in weight varied from 10 to 30 gm., the corrected weights after removal of the gastrointestinal tract ranged from 40 to 225 gm. There is necessarily an element of error no matter which procedure is followed but we believe that the injection of a constant dosage insures a more comparable method. In most of our experiments we have also determined the weights after the removal of the gastrointestinal tracts and have thus been able to analyze the results more satisfactorily.

Numerous experiments on relatively large numbers of animals are advisable in order to rule out individual variations in reactivity of "normal" animals. It is doubtful whether rabbits that have been infected with coccidia or have had snuffles or infected wounds are really normal in so far as the reactivity of macrophages is concerned. Furthermore, differences in nervous states, especially of the autonomic nervous system, as emphasized particularly by Müller (5) and Petersen (6), necessitate recognition of the importance of uniformity of handling, feeding and routine care so that reactivity may be as constant as is experimentally possible. In later stages of our work much less variation occurred, owing to the use of rabbits reared under exceptionally favorable conditions, and known to be absolutely free from "snuffles" and practically free from coccidial infection.

P. R. CANNON, F. L. SULLIVAN, E. F. NECKERMANN

The Passage of Staphylococci through the Lungs of Normal and Immune Rabbits

Inasmuch as opinions vary concerning the relative importance of the lungs in the removal of bacteria from the blood stream, it is desirable to know just how and in what numbers microorganisms pass through this area. To determine this, equal amounts of a suspension of *Staphylococcus albus*, usually from 1 to 3 cc. of a 24 hour broth culture, were injected simultaneously into the marginal ear veins of normal and of actively immunized rabbits. At exactly 2 minutes, 4 minutes, 5 minutes and at later intervals up to 1 hour, the lungs were fixed in Zenker-formol solution, sectioned and stained.

Fig. 1 illustrates the findings in a normal rabbit 2 minutes after intravenous injection. This is a camera lucida drawing of two pulmonary veins and shows the staphylococci evenly dispersed in the lumina with occasional ones engulfed by leucocytes. The bacteria are compact and well stained, show no evidence of any agglutinative tendency and are apparently passing rapidly and extracellularly through the lungs on their way to the general systemic circulation. In the immunized animal at this period there was no increased number of bacteria within the pulmonary tissues, and although polymorphonuclear leucocytes were present in greater numbers than normal, practically none contained staphylococci. In neither rabbit were mononuclear cells particularly active in phagocytosis of the microorganisms. Sections taken from the lungs of normal and immune rabbits 4 and 5 minutes and later after injection showed practically no extracellular microorganisms and only a few in polymorphonuclear leucocytes and macrophages.

It is evident from these experiments that the countless numbers of staphylococci in the venous blood stream pass almost instantaneously through the blood vessels of the lungs of both normal and immune rabbits. A comparatively small number are caught within the capillaries; this may be due to mechanical obstruction or chance collisions with leucocytes. The bacteria show no evidences of agglutination or swelling such as will be seen in the livers and spleens of these same rabbits. We conclude that under the conditions of our experiments the lungs play an insignificant part in "fixing" such antigen as staphylococci.

These results agree with those of Werigo (7) in his studies of anthrax in the rabbit, in which he found that within 8 minutes after intravenous injection numerous bacilli had been engulfed by polymorphonuclear leucocytes within the lungs, whereas the pulmonary macrophages played little if any part in the reaction. Within 3 hours, however, very few bacilli were seen in the lungs. Werigo believed that the microorganisms were momentarily caught mechanically, were phagocytosed by polymorphonuclear leucocytes and were then transported to the liver and spleen. On the other hand, Hopkins and Parker (2) observed that streptococci injected intravenously into cats were removed from the blood stream within from 10 to 30 minutes and were found most abundantly in the lungs, more being within macrophages than polymorphonuclear leucocytes. They observed no evidence of agglutination of the bacteria within the pulmonary tissues, neither in films of the blood, crushed preparations nor in sections. Their experiments with the injection of streptococci into rabbits, however, agree with Werigo in that the microorganisms tended to localize in the liver and spleen rather than in the lungs. Arima (1) also found very few live bacteria in the lungs of rabbits 5 minutes after the intravenous injection of *Staphylococcus aureus*, *B. coli* and *B. typhosus*. He assumed that they were either killed by the bactericidal action of the blood or were transported to other organs.

The Disappearance of Living Bacteria from the Blood Stream of Normal and Actively Immunized Rabbits

The relative rates of removal of living staphylococci and paratyphoid bacilli from the blood streams of normal and of actively immunized rabbits were determined by the methods described above. Thirty-two pairs of animals were tested. The characteristic response is shown in Chart 1, there being a uniformly accelerated rate of removal of the bacteria from the blood stream of the immune animal. There seems to be a distinct acceleration in rate of removal of the bacteria in the normal rabbit after from 15 to 30 minutes, but in most instances there are, comparatively, many more bacteria per cubic centimeter of blood in the normal animals, even as late as 75 minutes after injection. The time of complete disappearance was not determined, but most workers agree that practically all bacteria, regardless

of virulence, are primarily removed from the blood stream within a few hours.

The actual data from sixty of these rabbits are given in Tables I and II. Seventeen of the animals were immunized against *Staphylococcus albus* and compared with seventeen normal rabbits as to the comparative rates of removal of living staphylococci of the same strain, while thirteen others were immunized against *Bact. paratyphosum B* and similarly compared with thirteen normals. With the exceptions

CHART 1

Rate of disappearance of Staphylococcus albus from the blood stream of a rabbit (2a) immunized by a staphylococcal vaccine ----
12a (control) ———
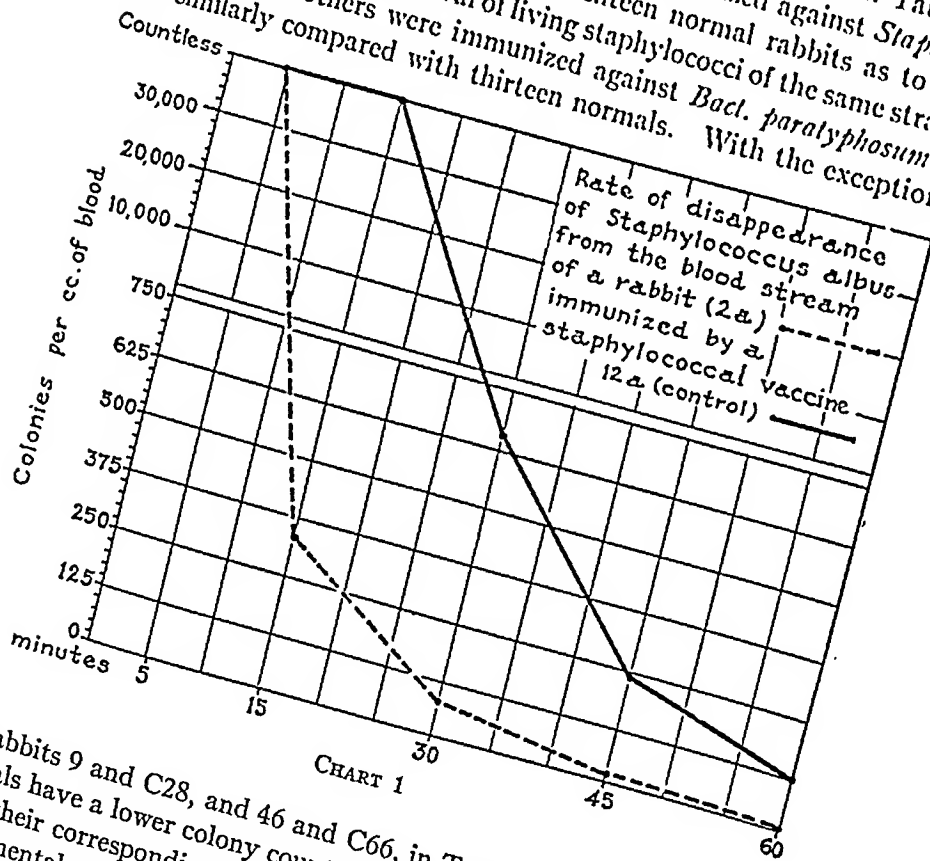
of Rabbits 9 and C28, and 46 and C66, in Table I, all of the immune animals have a lower colony count per cubic centimeter of blood than have their corresponding controls. These exceptions may be due to experimental error or to abnormal reactivity of the supposedly normal animals.

An analysis of the comparative weights of the animals after the removal of the gastrointestinal tracts (Table I) shows that eight of the immune rabbits weighed at least 20 gm. more than their controls while

TABLE I

The Disappearance of Staphylococci from the Blood Stream as Influenced by Previous Intravenous Immunization with a Staphylococcus Vaccine

Rabbit	Weight*		Time after termination of immunization	Time after intravenous injection, of cardiac puncture	Colonies per cc. of blood
	1	2			
	gm.		days	min.	
50	2,245–1,980		24	5	12,000
C70	2,290–1,875		—	5	Countless
5	2,240–1,700		15	16	590
C24	2,310–1,805		—	15	2,395
11	2,210–1,710		20	20	175
C30	2,165–1,690		—	17	1,400
12	2,250–1,780		20	19	20
C31	2,110–1,625		—	17	295
40	2,120–1,680		11	16	885
C60	2,150–1,715		—	15	990
41	1,985–1,590		11	15	117
C61	1,940–1,535		—	15	203
9	2,205–1,815		18	31	50
C28	2,195–1,730		—	30	28
10	2,090–1,615		18	33	27
C29	2,020–1,695		—	32	655
42	2,535–2,010		15	32	30
C62	2,545–2,110		—	30	83
43	1,800–1,355		14	30	41
C63	1,780–1,345		—	30	75
7	2,005–1,650		16	35	100
C26	1,980–1,475		—	35	182
8	2,400–1,765		16	47	31
C27	2,345–1,600		—	45	43
1	1,900–1,520		12	63	11
C20	1,930–1,325		—	62	120

* 1 = original weight. 2 = weight after removal of gastrointestinal tract.

TABLE I—Concluded

Rabbit	Weight		Time after termination of immunization	Time after intravenous injection of cardiac puncture	Colonies per cc. of blood
	1	2			
	gm.		days	min.	
2 C21	2,225-1,715		13	61	
	2,220-1,710		—	62	16
46 C66	2,315-1,870		18	62	29
	2,310-1,740		—	62	49
3 C22	2,150-1,720		14	60	10
	2,195-1,810		—	123	
47 C67	2,225-1,835		23	122	2
	2,195-1,860		—	hr. 24	45
				24	0
					0

five normal animals weighed more than their immune partners in the experiment. Nevertheless, the trend in all pairs, except as above noted, was toward the more rapid elimination of the bacteria from the blood streams of the immune animals. Therefore the variations in weight have apparently not affected the comparison of bacterial elimination following the use of a constant dosage of bacteria for each pair of animals tested.

These experiments were done at time periods varying from 6 to 46 days after the termination of immunization but no significant differences were evident within this range. Presumably the optimal period should be from 7 to 21 days, assuming that the disappearance rates are dependent upon the union of sensitized macrophages with well opsonized bacteria.

The question arises as to whether this accelerated rate of removal may not be the result of non-specific stimulation resulting from immunization rather than from specific causes. We have evidence, to be reported in a later paper, that non-specific stimulation may, under certain conditions, accelerate the removal mechanism, but in general, we are convinced that specific active immunization more effectively facilitates the removal of the bacteria injected. Thus, Chart 2 illustrates the type of curve obtained when animals immunized against staphylococci were tested as to their ability to eliminate *Streptococcus*

TABLE II

The Disappearance of Bact. paratyphosum B from the Blood Stream as Influenced by Previous Intravenous Immunization with A. Bact. paratyphosum Vaccine

Rabbit	Weight* 1 2		Time after termination of immunization	Time after intravenous injection, of cardiac puncture	Colonies per cc. of blood
	gm.		*days*	*min.*	
47	1,735–1,250		7	15	86
C58	1,655–1,280		—	15	141
48	2,235–1,730		7	15	52
C59	1,955–1,390		—	15	525
49a	2,005–1,970		9	33	535
C59a	2,100–1,965		—	33	3,370
45	1,315– 910		6	65	9
C56	1,370– 975		—	65	45
46	1,570–1,070		6	65	2
C57	1,550–1,180		—	65	9
1	2,060		8	65	27
C14	2,050		—	65	87
5	1,865–1,415		15	65	16
C18	1,695–1,340		—	65	41
30	2,055–1,610		10	92	5
C50	2,070–1,570		—	92	205
41	2,275–2,040		10	125	5
C51a	2,105–1,940		—	125	56
42	2,365–1,990		12	123	3
C52	2,250–1,705		—	123	14
9	2,345–1,895		46	135	1
C21	2,330–1,840		—	125	15
2	1,825–1,430		11	260	6
C15	1,800–1,285		—	260	10
3	1,810–1,310		11	325	10
C16	1,845–1,305		—	325	330

* 1 = original weight. 2 = weight after removal of gastrointestinal tract.

hemolyticus from the blood stream. As is seen, the curves for the immune and normal animals are practically identical; in fact, in the two animals charted, the immune reacted less effectively than the normal and died on the following day.

It is evident, then, that active immunization changes the reactivity of rabbits in the elimination from the blood stream of certain living bacteria. Further analysis shows that the bacteria are removed and
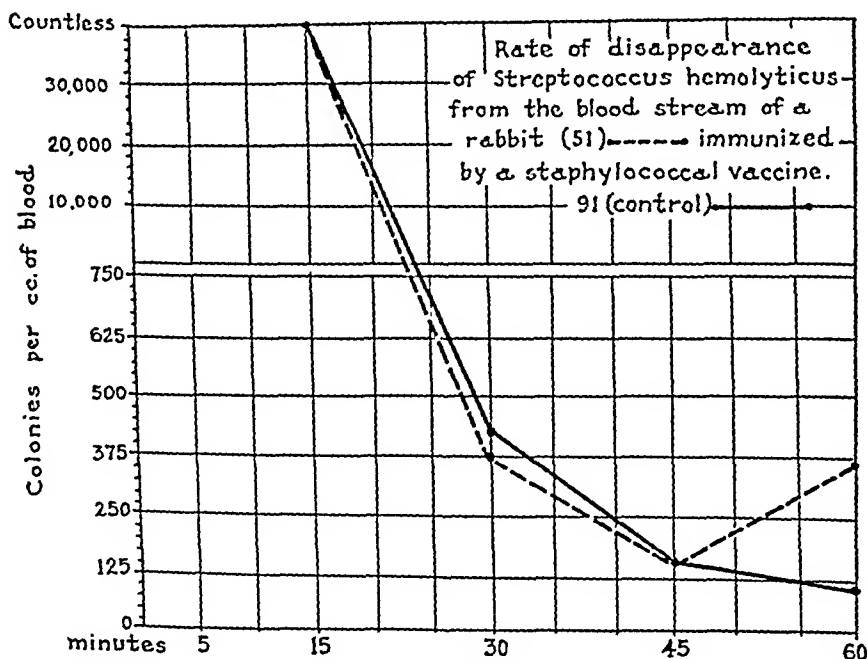
CHART 2

"fixed" in various organs and tissues, particularly in the liver and spleen, where they are killed and digested.

The Mechanism of Removal of Bacteria from the Blood Stream by the Liver and Spleen

The microscopical examination of sections from the livers and spleens of these rabbits revealed an enormous concentration of the bacteria in the immune animals with unmistakable evidence of an increased degree of disintegration of the microorganisms themselves.

It was apparent that some immunity mechanism was in action and that in all probability the more rapid removal of the bacteria from the blood stream in the immune animals was caused by the mechanism which was facilitating their fixation. The most striking feature, however, was the extraordinary speed of the process in the immune animals, astonishing changes occurring within 2 minutes after the intravenous injection of the staphylococci. Fig. 2 illustrates this effect in the spleen. It is a composite drawing, made by the aid of a camera lucida, of four oil immersion fields from smears of splenic pulp fixed by methyl alcohol and stained by Giemsa stain. These smears were made from the spleens of two rabbits, a normal and one previously immunized against the staphylococcus, exactly 2 minutes after the simultaneous injection into the marginal ear veins of each rabbit of equal quantities of the same suspension of staphylococci. In the smear from the normal rabbit there are relatively few bacteria, these are individually distributed, seem to be extracellular and are only slightly swollen. In the immune animal, however, there are many staphylococci which occasionally occur in clumps or masses with the individual microorganisms swollen and more faintly stained than are those in the normal rabbit. Evidently antibacterial substances have almost instantaneously affected the bacteria so that they have become swollen, seem to have an increased cohesiveness or decreased viscosity and stain relatively poorly. The bacteria appear to be extracellular, but splenic cells may have been ruptured in making the smears. At any rate, their concentration in the immune spleens and the extraordinarily rapid changes in their morphology are striking.

The spleens from these animals were fixed immediately in warm Zenker-formol solution, sectioned, and stained by the Claudius-Gram method. Figs. 4 and 5 are photomicrographs from them. In the normal spleen, as in the smears, there are relatively few staphylococci per oil immersion field, but, although the bacteria are distinctly stained, they are already somewhat swollen and are, for the most part, within macrophages. In the immune spleen, however, there are vast numbers of staphylococci per oil immersion field, they are indistinctly stained, are markedly swollen and are almost entirely within macrophages.

Similar changes occurred in the livers of animals examined 5 minutes

after injection of equal quantities of a suspension of staphylococci. In the normal animal an occasional staphylococcus was seen in Kupffer cells, whereas, in the immune animal, many large, swollen cocci were adherent to the walls of the sinusoids as well as within swollen Kupffer cells (Fig. 3). Many of the extracellular cocci seemed flattened as though larger portions of their surfaces were in contact with the lining membrane of the sinusoid.

At later stages sections from the spleens and liver have consistently shown a similar picture of greatly increased concentration and more marked phagocytosis of the bacteria in the immune organs. Furthermore, the ingested bacteria lose their staining properties and disappear much more rapidly in the spleens and livers of the immune animals, in spite of their greater concentration in such organs.

DISCUSSION

These experiments demonstrate the extreme rapidity with which certain reactions may occur within the immune body and suggest an important correlation between antigen and sensitized macrophages *in vivo*. The fact that the accelerated rate of removal of bacteria from the blood stream is dependent upon specific immunization indicates that neither the mere increase in macrophages nor their stimulation by non-specific agents is solely responsible for the clearance of bacteria from the blood. Furthermore, the marked changes in the size and degree of staining of the bacteria in the immune livers and spleens and their greater concentration there, suggest that the immune bodies probably act most effectively in the organs in which macrophages are most numerous. On the other hand, the presence of immune bodies in the blood stream does not determine bacterial localization throughout the tissues generally since the bacteria do not tend to become localized in the lungs, muscles, etc., and when seen there, they do not show the pronounced morphological changes observed in the immune livers and spleens.

We interpret the general mechanism as follows: Staphylococci injected intravenously into normal rabbits circulate throughout the blood stream in large numbers, probably making many passages through the organs and tissues of the body. In passing through the spleen and liver, especially, conditions more favorable for phagocy-

tosis may obtain, particularly those dependent upon slow blood flow, availability of macrophages and leucocytes, mechanical conditions favoring filtration, etc. Chance contacts between phagocytic cells and the relatively unchanged staphylococci induce a certain degree of phagocytosis, as seen in the polymorphonuclear leucocytes in the lungs and in the macrophages and leucocytes of the liver and spleen. Eventually this mechanism removes the bacteria from the circulating blood. Their further fate doubtless depends upon the virulence of the microorganisms and the digestive capacities, or in other words the functional state of the phagocytes, both macrophages and polymorphonuclear leucocytes. As Werigo showed in experimental anthrax infection, if these cells become inadequate, the bacteria again multiply and generalize.

In the immune animals, however, this normal mechanism disposed of the dead bacterial bodies during the preliminary period of immunization, at which time many macrophages in the liver and spleen removed and digested the bacterial particles. When at a later period, large numbers of living staphylococci run the gauntlet of these macrophages, there is an almost instantaneous swelling of the microorganisms, an increasing obstruction to their free passage through the immune liver and spleen and a tendency to clumping of the bacteria, with a resulting retention of such affected microorganisms in these organs.

The presence of swollen, clumped, poorly stained bacterial bodies suggests a marked alteration in their physical state, such as a decreased viscosity or increased cohesiveness, and the significance of the morphological changes may be explained by the experiments of Mudd and his associates (8) on the effects of immune bodies upon antigen. At any rate, it is apparent that the bacteria have been more effectively "fixed" and destroyed than under normal conditions.

The observations of Manwaring and Coe (9) merit renewed attention with respect to the above findings. These workers observed that pneumococci suspended in sterile Ringer's solution were removed rapidly in the livers of actively immunized rabbits when the organs were perfused *in situ* through the portal vein, whereas comparatively few bacteria were removed in normal livers under similar conditions. They reported that "smears and histological preparations made from

the perfused liver now show numerous pneumococci adherent to the capillary endothelium. Few if any agglutinated masses are seen, and little or no endothelial phagocytosis." After demonstrating that immune serum added to the perfusing fluid caused a similar effect whereas normal serum did not, they suggested the name "endothelial opsonin" for the serum component responsible for the retention of the pneumococci. The significant fact, however, is their demonstration of the bacteriotropic action leading to an increased degree of cohesion of the microorganisms to the lining membrane of the hepatic sinusoids, whereby the antigen is fixed and later engulfed and destroyed by the hepatic macrophages.

Hopkins and Parker (2) apparently observed a somewhat similar adherence of streptococci to the walls of capillaries of the lungs in cats injected intravenously with streptococci and in which the lungs were perfused 30 minutes later with salt solution and Helly's fluid. They stated that the streptococci were not washed out, were not with- in leucocytes but were, for the most part, either in large mononuclear cells or in an eosin-staining matrix which they thought might represent a section through endothelial protoplasm or some substance from the blood deposited around them. More recently Rich (10) has suggested a similar mechanism to explain the fixation of pneumococci introduced into the skins of rabbits previously injected with immune serum. He concluded that "local fixation appears to be effected primarily by a prompt and specific agglutination of the bacteria which impedes their free movement through the tissues" and which "appears to involve a phenomenon of adsorption to the tissues as well."

Bull (11, 12) has emphasized the importance of agglutination *in vivo* in the mechanism of removal of bacteria from the circulating blood, his opinion being that the microorganisms are agglutinated in the blood stream and removed by the polymorphonuclear leucocytes, especially in the liver and spleen. As the clumps of bacteria observed in his experiments were in smears from the blood and tissues, it is possible that they were formed, in part at least, in the process of mak- ing the smears. Zinsser (13) and Rheingold (1) have also presented evidence supporting the conception of agglutination in the circulating blood as a mechanism of defense. The latter observer described aggregates of *B. prodigiosus* in the capillaries and smaller vessels in the

livers of dogs injected intravenously with this microorganism, although he found no evidences of such a process in blood smears from the peripheral blood.

In our experiments we have at no time obtained any clear-cut evidence of such a phenomenon in the liver, spleen or lungs of normal or immune animals, although there may be a slight suggestion of such in Fig. 2. The small clusters of staphylococci may at least be regarded as evidence of an increased cohesiveness of the bacteria, but here again the clumps may have been formed in making the smears. No such indication of agglutination was apparent in the sections of the spleens and livers of these animals. As noted above, Manwaring and Coe observed no marked agglutination in the perfused livers, either normal or immune, nor did Hopkins and Parker in the tissues of cats and rabbits injected with streptococci.

Nevertheless, the absence of observable agglutination *in vivo* under the above conditions does not prove that such a process may not be of considerable importance as a means of localizing bacteria. The time factor may be the determining element. For instance, if immune tissues are so changed that bacteria are practically instantaneously arrested and then engulfed by phagocytes, obviously no agglutination could occur, as no opportunities for collisions between bacteria would be present. There is evidence, however, that when staphylococci remain extracellularly in immune tissues for a sufficient length of time, agglutination *in vivo* actually occurs, presumably because of the bacteriotropic effect of immune bodies in increasing the cohesiveness of the bacteria (14).

In whatever manner the bacteria may be fixed in the tissues, whether by actual adherence to the surfaces of normal cells or tissues, or by an agglutinating or agglomerating action whereby their dispersion through the tissues may be impeded or minimized, to that extent their generalization is limited and the dangers of disseminated infection lessened.

The relative importance of macrophages and polymorphonuclear leucocytes in the mechanism as a whole is of considerable interest. In such a complex system, however, it is questionable whether too much emphasis should be placed upon the comparative significance of two groups of mesenchymal cells whose functions are so similar

and apparently complemental. In the passage of staphylococci through the lungs many are unquestionably engulfed by polymorphonuclear leucocytes, although for the most part they pass quickly through and become generalized. In the liver and spleen, however, the bacteria are rapidly fixed by macrophages, although here, too, some are phagocytosed by polymorphonuclear leucocytes, which in turn are later engulfed by macrophages. We have repeatedly observed macrophages in the spleen containing many cocci side by side with polymorphonuclear leucocytes containing none. Apparently the primary reaction is mainly between the cocci, immune bodies and the cytoplasmic surfaces of the macrophages, accompanied or quickly followed by the accumulation of polymorphonuclear leucocytes attracted or retained there by chemotactic or electrotropic influences. Both types of cells then engulf the bacteria and destroy them provided that the virulence and numbers of the latter are not too great.

CONCLUSIONS

1. The simultaneous intravenous injection into normal and actively immunized rabbits of equal quantities of living staphylococci or paratyphoid bacilli is followed by a distinctly accelerated rate of removal of the bacteria from the blood streams of the immune animals.

2. This altered reactivity is due essentially to specific active immunization.

3. The bacteria pass rapidly through the capillary bed of the lungs, extracellularly and dispersed for the most part, and become generalized through the blood stream.

4. The bacteria are quickly removed from the circulating blood in the immune animals and less rapidly in the normal ones, by various organs, particularly the liver and spleen, where they accumulate in enormous numbers, become adherent to the lining membrane of the sinusoids of the liver and apparently to the macrophages of the spleen and are phagocytosed by the macrophages and leucocytes in these organs.

5. Associated with this effect are morphological changes in the bacteria as shown by swelling, loss of staining power and evidences of increased cohesiveness and decreased viscosity, these changes being apparent as early as 2 minutes after their intravenous injection.

6. Inasmuch as these changes are not seen to a marked degree within the lungs or other organs, they are probably the result of a local antigen-antibody reaction of a bacteriotropic type in the two organs generally considered to be most actively concerned with the production of immune bodies.

7. By means of this accelerated bacteriotropic effect in the actively immunized animals, phagocytosis is facilitated and intracellular digestion of the bacteria is enhanced.

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EXPLANATION OF PLATES

PLATE 5

FIG. 1. Drawing made with the camera lucida (×820) of two pulmonary veins from a normal rabbit which had been injected intravenously in the marginal ear vein 2 minutes previously with a heavy suspension of staphylococci.   The micro-

organisms are mostly extracellular, are dispersed, of normal size and are well stained. An occasional polymorphonuclear leucocyte has engulfed staphylococci.

Fig. 2. Drawing made with the camera lucida of smears of splenic pulp from two rabbits injected simultaneously intravenously with equal quantities of the same suspension of staphylococci (×820). The smears were made 2 minutes after the injection, were fixed immediately by methyl-alcohol and stained by the Giemsa stain. Each picture is a composite of four oil immersion fields made under identical optical conditions. In the smear of the normal rabbit's spleen (a) there are comparatively few staphylococci, which are dispersed, are of practically normal size and are well stained. In the smear from the immune animal (b) there are many staphylococci, which are swollen, poorly stained and in places are in small clusters.

Fig. 3. Drawing made with the camera lucida (×820) of an hepatic sinusoid from an actively immunized rabbit 5 minutes after the intravenous injection of a heavy suspension of living staphylococci. The liver was perfused with sterile solution of sodium chloride followed by warm Zenker-formol solution. Note the adherence of the swollen microorganisms to the wall of the sinusoid although the erythrocytes have been washed out by the perfusing fluids.

PLATE 6

Fig. 4. Photomicrograph (×970) of a section of spleen from the normal rabbit described in Fig. 2. The spleen was fixed 2 minutes after the intravenous injection of the staphylococci and the section stained by the Claudius-Gram stain. Note the dispersed but well stained staphylococci, many of which, however, are swollen and either adherent to or within macrophages. Only a few polymorphonuclear leucocytes are present.

Fig. 5. Photomicrograph (×970) of a section of spleen of the staphylococcus-immune rabbit described in Fig. 2. The spleen was fixed 2 minutes after the intravenous injection of the staphylococci and the section stained by the Claudius-Gram stain. Note the marked concentration of staphylococci, the swollen as well as fading forms and the accumulation of polymorphonuclear leucocytes.

PLATE 5
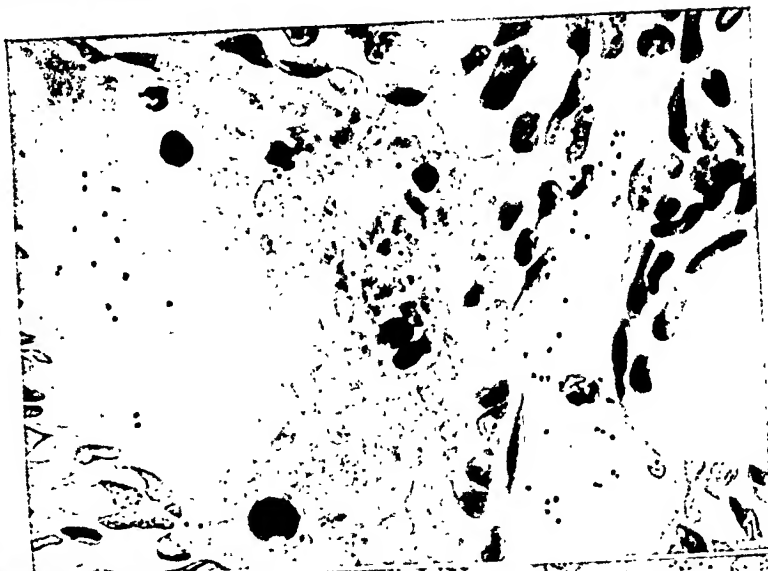
(Cannon *et al.*: Disappearance of bacteria from blood stream)
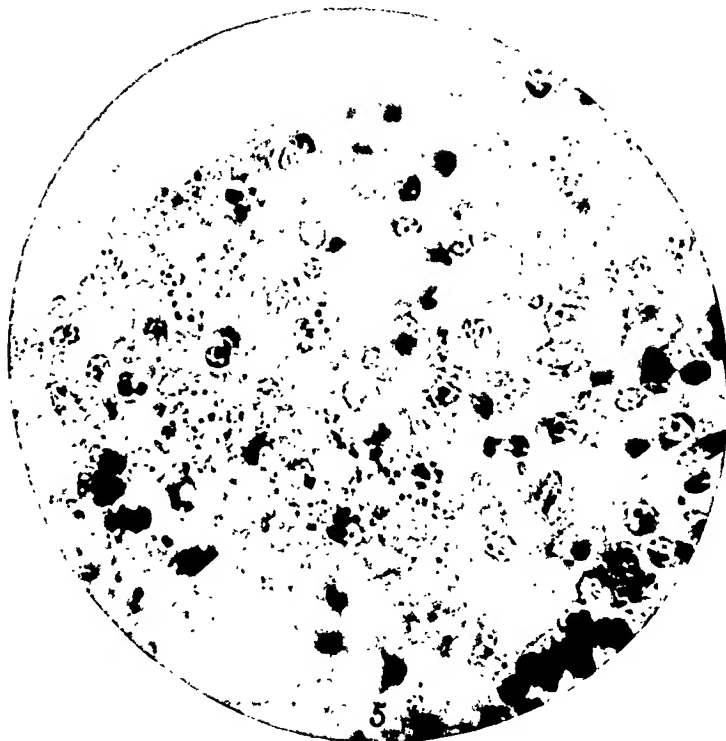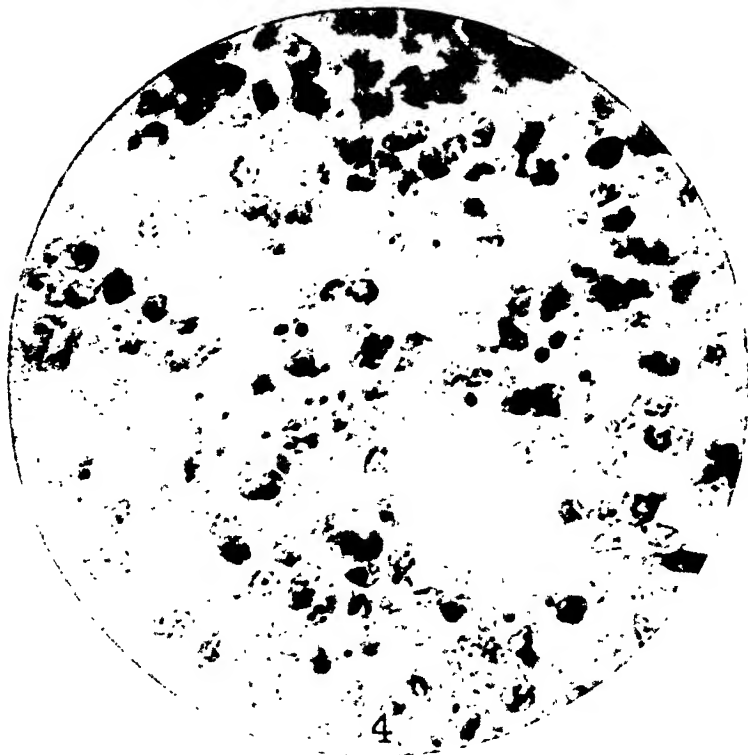
(Cannon *et al.*: Disappearance of bacteria from blood stream)

THE INFLUENCE OF AGE AND OF DURATION OF TREATMENT ON THE PRODUCTION AND REPAIR OF BONE LESIONS IN EXPERIMENTAL HYPERPARATHYROIDISM*

By HENRY L. JAFFE, M.D., AARON BODANSKY, Ph.D., and JOHN E. BLAIR, Ph.D.

(From the Laboratory Division of the Hospital for Joint Diseases, New York)

PLATES 7 TO 9

We have pointed out that young guinea pigs weighing between 280 and 370 gm., when injected subcutaneously with a single dose of 20 units of parathormone per 100 gm. of body weight, develop severe and rapid decalcification of the skeleton, which becomes progressively more intense up to about the 48th hour. In contrast to these findings, the skeletons of adult guinea pigs weighing between 620 and 860 gm., and injected with the same doses, do not show histological changes. The serum calcium and phosphorus, however, indicate effects of parathormone on the adult as well as on the young guinea pigs, although they are pronounced in the latter (1, 2).

The striking difference in the histologic appearance of the skeletons of young and adult guinea pigs given single doses of parathormone led us to study in greater detail the effect of parathormone on guinea pig bones as influenced by the age of the animal. Furthermore, we studied the influence of increasing age on the effects of repeated or intermittent doses in young and old guinea pigs, as reflected in bone changes. This permitted us to make observations concerning the so called "immunity" to repeated doses of parathormone, which is supposed to develop. The details of our experimental methods are given in the papers mentioned above.

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Criteria of Age

In healthy stock the age of a growing guinea pig is well indicated by its weight. Our animals weigh between 70 and 90 gm. at birth; between 200 and 260 gm. at 1 month; between 300 and 400 gm. at 2 months; between 425 and 525 gm. at 3 months; between 500 and 600 gm. at 4 months. After this, increase in weight is much slower, our animals weighing between 600 and 700 gm. at the age of 6 months and between 800 and 1000 gm. at about 1 year. We consider a guinea pig fully adult when the epiphyseal cartilage plates of the upper end of the humerus, the lower end of the femur, and the upper end of the tibia are closed (they need not have disappeared). At this time the cells of the epiphyseal cartilage plate no longer retain their regular columnar arrangement, while the metaphyseal surface of the plate is becoming bridged by bone. From our casual observations, we believe that closure occurs at about the 10th month. As the animal becomes older, these closed epiphyseal cartilage plates become thinner and eventually disappear. While we have made no systematic study, our observations have led us to conclude that this does not take place in these plates until some time after the 1st year. This is quite in line with what Dawson showed for the albino rat (3)—that these epiphyseal cartilage plates do not disappear until senility. Guinea pigs between the 4th and 9th month of age may be considered young adults. Sexual maturity, however, occurs quite early in our stock. We have on several occasions observed pregnancy of approximately 1 to 2 weeks' duration in animals about 2 months of age.

EXPERIMENTAL

The Relative Absence of Parathormone Effects in the Bones of Guinea Pigs Treated Daily to the Age of 110 to 120 Days

In four guinea pigs 2 to 7 days of age, weighing between 78 and 128 gm., the injection of 1 or 2 units of parathormone daily was begun. This dose was gradually increased, the animals receiving 10 units a day after 16 days of treatment. They almost doubled their original weight during this period. The dose was then increased by steps to 20 units daily, one animal being given this dose on the 23rd day of the experiment, and the others on the 36th day. The animals then weighed 325, 296, 281, and 269 gm., as compared with 319, 290, 269, and 235 gm. respectively for their untreated litter mate controls. The dose of 20 units daily was continued for 65 days in one animal, 73 days in two, and 87 days in the fourth. The animals received total doses of 1500 to 1890 units of parathormone during the course of treatment. However, we do not consider the total dose as significant as the size and the gradation of the doses, and as the duration of treatment. The guinea pigs gained consistently in weight throughout the experiment.

The animal which had been injected with 20 units daily for 65 days weighed 508 gm. when its litter mate control reached 519 gm. Both were then fasted for 48 hours and killed to terminate the experiment. The animals receiving 20 units

of parathormone daily for 73 and 87 days, weighed 443, 560, and 497 gm., respectively; their controls 579, 553, and 482 gm. All were killed 5 days after the last injection of parathormone at which time they were 115 to 120 days old. They showed no hypercalcemia at the termination of the experients, but on the contrary, a hypocalcemia, the significance of which is discussed elsewhere (4).

The bones of the test animals showed a surprising absence of severe changes. Those treated for 65 days showed slight to moderate resorption of the cortices of the ribs and long bones, indicated by enlargement of the vessel canals, which contained young connective tissue. Except in the metaphyses of the upper end of the tibia and the lower end of the femur there was no marrow fibrosis. These metaphyses showed numerous irregular trabeculae of cartilage with osteoid borders, surrounded by narrow zones of bone lined by osteoblasts. Some osteoid was present in the connective tissue between the trabeculae. The epiphyses showed nothing unusual; the trabeculae were thick, regular, and lined by osteoblasts. The epiphyseal cartilage plates and the costochondral junctions were as yet not closed.

The three animals treated for 73 and 87 days with 20 units of parathormone daily showed even fewer bone changes. There was at most a slight thinning of the cortices, especially of the ribs, and slight widening of some of the vessel canals, which contained young connective tissue. There was no metaphyseal marrow fibrosis, endosteal resorption, or abnormal phagocytosis by osteoclasts. The epiphyses showed a complete absence of resorption, the trabeculae being quite thick and lined by osteoblasts. The epiphyseal cartilage plates were not closed.

This group of guinea pigs brought out strikingly the relative absence of parathormone effects upon the bones as a result of daily injections to the age of 110 to 120 days. On the basis of our previous experience (1), we believe that after receiving 20 units of parathormone daily for 2 or 3 weeks, the bones of these animals had undergone severe decalcification, but when this treatment was continued, its effects, as indicated by bone changes, diminished. In spite of further continuance of the treatment with the same dose of parathormone, healing of the earlier lesions was possible (Figs. 1 and 2).

The Relative Absence of Parathormone Effects upon the Bones of Guinea Pigs Treated with Intermittent Injections of Large Doses of Parathormone to the Age of 95 to 145 Days

Three guinea pigs about one week old, weighing 100, 110, and 150 gm., were injected daily for 16 days with small, gradually increasing doses of parathormone. They were first given 2 units daily and finally received 10 units daily. At the end of this period the animals weighed 190, 220, and 240 gm., respectively. They were rested for 6 days, at the end of which time they weighed between 230, 250,

and 270 gm. Intermittent injections of large doses of parathormone were then begun, the animals being a month old. The injections were given at intervals of from 7 to 11 days; on one occasion, in two animals, 15 days elapsed between injections, during which period the animals had been bled from the heart to determine the serum calcium and phosphorus.

One guinea pig was given its first large subcutaneous injection of 60 units of parathormone when it weighed 250 gm. The intermittent injections covered a period of 66 days, and a total of 800 units were given. The animal was found dead 48 hours after the last dose (140 units). It was then 95 days of age, and weighed 370 gm. Death was due to acute hyperparathyroidism.

Autopsy showed beading at the costochondral junctions. The bones were soft. On histological examination, all of those examined showed moderate resorption; the Haversian canals were enlarged and contained young connective tissue with osteoclasts in Howship's lacunae. The marrow of the metaphyses was scarred, but osteoid tissue was absent here and elsewhere. Along the inner surface of the cortices, especially near the costochondral junctions and the epiphyseal cartilage plates, there were some Howship's lacunae and osteoclasts. At the costochondral junctions and in the metaphyses, areas of more acute decalcification were observed; these acute changes were probably due to the dose of 140 units of parathormone injected 48 hours before death. It should be noted that this animal was only 95 days of age, and that the epiphyseal cartilage plates were open. The effects of the previous intermittent doses were indicated by the numerous distinct longitudinal cement lines, and, where Haversian canals were present, by irregular cement lines, arranged in relation to the canals, giving a mosaic appearance to the bone.

Two guinea pigs weighed 270 and 230 gm., respectively, when intermittent injections were started. The first doses were 60 units and the final doses 140 units. The guinea pigs received 1420 units of parathormone each. 4 days after the last injection, the animals were killed to terminate the experiment. They weighed 675 and 540 gm. respectively and were about 20 weeks old.

On histological examination, the cortices of the ribs and long bones were found to be compact. The vessel canals were, at most, only slightly enlarged, and contained no significant increase of connective tissue. The marrow of the long tubular bones, even in the metaphyses, was not fibrosed. The epiphyseal cartilage plates showed the earliest indications of closing. Many ribs were beaded at the costochondral junctions, due to new bone formation subsequent to fractures in these regions. The compact and spongy bone showed marked accentuation and increase of the cement lines. These ran parallel, but in the cortices of the long bones they were irregular, giving the bone a mosaic appearance. The innumerable cement lines were the only indication of the effects of the earlier injections of parathormone, the fractures at the costochondral junctions being probably due to the last one or two doses.

The age factor influencing the effect of parathormone on the bones of guinea pigs is also brought out in this group. The animal 95 days of age at autopsy, although it had received only slightly more than one-half of the amount of parathormone given to the others, succumbed to a dose of 140 units, and its bones showed some of the effects of acute hyperparathyroidism. The other two animals were 140 and 145 days of age. They showed increasing ability to withstand the injection of large doses of parathormone, and were not killed by several successive doses of 140 units. Their bones showed relatively very little decalcification (Figs. 3 to 8). All of these animals showed the effects on the bones of intermittent injections of parathormone. These were indicated by the numerous cement lines which gave a mosaic appearance to the bones (Fig. 6).

The Effect of Extremely Large Single or Pyramided Doses of Parathormone on the Bones of Adult Guinea Pigs

We have already shown that the subcutaneous injection of large doses of parathormone (20 units per 100 gm. of body weight) into adult guinea pigs weighing from 620 to 860 gm. resulted in no appreciable changes in their bones, which were examined up to 48 hours after the injection (1). In attempting to produce bone changes with parathormone in adult guinea pigs, it was therefore decided to use much larger doses (5).

A male guinea pig weighing 900 gm. was injected subcutaneously with 100 units of parathormone per 100 gm. of body weight. It received a total of 900 units of parathormone (45 cc.) given in two equal doses within a period of 2¼ hours. For 48 hours after the injection the animal did not appear ill. At the end of this period it was killed. The serum calcium and phosphorus were 15.7 and 4.9 mg. per 100 cc. respectively. Blood urea nitrogen was 9.2, and chlorides 489 mg. per cc. Hemoglobin was 14.8 gm. per 100 cc.

At autopsy the bones were dense and resisted cutting. The liver contained one large and several small areas of anemic necrosis. The heart showed some degeneration of the muscle fibers. When stained by the von Kossá method, considerable calcium was found in the necrotic areas of the liver. On histological examination the bones showed strikingly few changes. The cortices of the ribs and of the long tubular bones were compact; the vessel canals were of normal diameter. Two of the ribs showed small areas of acute decalcification in the vicinity of the costochondral junctions, and here a few osteoclasts were found.

The epiphyseal cartilage plates of the long tubular bones were closed and the marrow and trabeculae of the metaphyses showed nothing unusual. The trabeculae and marrow of the epiphyses also were normal. There was no osteoclastic resorption here.

The experience gained from this animal led us to investigate the effects of still larger single doses.

An adult female guinea pig weighing 1040 gm. was injected with 2080 units (104 cc.) of parathormone, on the basis of 200 units per 100 gm. of body weight. The parathormone was given subcutaneously in two equal doses at an interval of 3 hours. 24 hours later the animal was very ill; it died 40 hours after the injection.

Autopsy showed considerable edema of the fat and subcutaneous tissues. The lungs were congested; pneumonia was absent; the heart was whitish and contracted; the suprarenals were congested; the liver, spleen, and kidneys showed nothing unusual in the gross. Histological examination showed besides the edema scattered necrosis in the subcutaneous tissues. There was no degeneration of the kidney tubules, nor of the liver. Calcium was found on histological examination in greatest amounts in the lung, and to a less extent in the other tissues.

Sections of the bones (Figs. 9 and 10) showed a striking absence of parathormone effects. The cortices of the ribs and long tubular bones were compact. The vessel canals were small, and there was no pathological subperiosteal or subendosteal osteoclastic resorption. The epiphyseal cartilage plates of the long tubular bones were closed, as were the costochondral junctions of the ribs. The rib cartilages showed beginning ossification. The skull and jaw bones also showed no pathological resorption or fibrosis. The marrow, especially near the costochondral junctions and in the vicinity of the closed epiphyseal cartilage plates, was congested, but there was no marrow necrosis. The trabeculae and marrow of the epiphyses were normal. This animal was older than the last one discussed, and twice the dose was therefore ineffective in producing bone changes, although the animal was killed by the general toxic effects of the parathormone.

Still testing the possibility of altering seriously the histological structure of the bones of an adult guinea pig, we injected an animal with a total of 300 units of parathormone per 100 gm. of body weight, in doses pyramided over a period of 48 hours. An adult male guinea pig weighing 860 gm. was injected with three doses of parathormone (860 units each), at intervals of 24 hours between the doses. The animal thus received a total of 2580 units (129 cc.) of parathormone within 48 hours. The sites of injection were varied to assure absorption.

24 hours after the last injection, blood was withdrawn from the heart, under ether anesthesia, for chemical analyses. The serum calcium and phosphorus were 19.6 and 4.7 mg. per 100 cc., respectively. About 48 hours after the last injection, and at the end of the 4th day of the experiment, the animal was killed when moribund. It weighed 860 gm. The serum calcium and phosphorus were 18.9 and 8.4 mg. per 100 cc., respectively, the urea nitrogen 40 mg. and sodium chloride 430 mg. per 100 cc. The hemoglobin was 14.6 gm. per 100 cc.

At autopsy the liver showed several patches of necrosis, all within an area of 8 mm. The bones resisted cutting. On histological examination, the liver cells showed cloudy swelling; nothing unusual was seen in the kidneys aside from necrosis of a few tubules. The other organs showed nothing unusual and contained no large deposits of calcium. A section from tissues at one of the sites of injection showed edema and necrosis of both the connective tissue and muscle, and calcium was demonstrated by the von Kossá method.

On section the cortices of the long tubular bones were quite compact, the vessel canals showing only slight enlargement. Underneath the periosteum and endosteum there were numerous Howship's lacunae filled with osteoclasts. The epiphyseal cartilage plates of the long bones were not completely closed, the cartilage cells still preserving, to a great degree, the columnar arrangement. So severe was the decalcification that epiphyseal separation had occurred in all the long tubular bones. The metaphyses showed, as would be expected, a larger number of Howship's lacunae with osteoclasts on the surfaces of the bony trabeculae. There was in addition slight scarring of the metaphyseal marrow. On the surface of the cortices of the ribs were Howship's lacunae with osteoclasts, more abundant on the subendosteal than on the subperiosteal surface. The vessel canals of the ribs were slightly enlarged. Some acute decalcification and marrow fibrosis was observed at the costochondral junctions. The skull and jaw bones also showed resorption, with Howship's lacunae and osteoclasts in abnormal numbers. Some resorption of the trabeculae of the epiphyses with osteoclasts and Howship's lacunae was observed.

This guinea pig, from the appearance of its epiphyseal cartilage plates, and from the fact that epiphyseal separation had occurred, was younger than the first two. It is therefore all the more significant that in this animal, probably not fully adult, (see page 140) 2580 units of parathormone, pyramided within a period of 48 hours, were required to produce moderately severe bone resorption. However, the bone changes were less than those produced by much smaller doses in younger animals (Figs. 10 and 12).

The Effect of Continued Daily Injections of Parathormone on the Adult Guinea Pigs

We have demonstrated that the injection of large single doses of parathormone into two adult guinea pigs produced no effects on the bones of the animals and that in one case when 2580 units were administered, some changes had been produced. We decided to ascertain the effects of repeated doses of parathormone on adult animals.

TABLE I

Summary of Daily Treatment of Adult Guinea Pigs

Day of experiment	No. 1206, M.		No. 1195, M.		No 1135, F.		No. 959, F.		No. 1136, F.		No. 1196, M.		No. 1194, M.	
	Weight	Dose	Weight	Dose	Weight	Dose	Weight	Dose	Weight	Dose	Weight	Dose	Weight	Dose
	gm.	units	gm.	units	gm.	units	gm.	units	gm.	units	gm.	units	gm.	units
1	890	20	950	20	900	20	1015	20	1000	20	820	20	950	20
2		20		20		20		20		20		20		20
3		20		40		40		40		40		20		40
4		20		40	820	40		40	990	40		40		40
5		40	960	60		60	965	60		60	770	40	860	60
6	830	40		60		60		60		60		60		60
7	830	40	880	60		60		60		60		60		60
8					750	60		60		60		60		60
9							840	60	990	60		60		60
10									970	60		60		60
11											620	60	750	60
12												0		
13											580	0		
Total units...		200		300		360		420		480		500		540

Seven adult guinea pigs received daily injections of parathormone for 7 to 11 days. They received a total of from 200 to 540 units as shown in Table I. All died from acute hyperparathyroidism.

The histological examination showed that with such treatment bone and marrow changes could be produced in adults; the degree varied directly with the total dosage of parathormone given, but the adults which were younger showed relatively more bone and marrow change, on a given dosage, than those that were older. For example, No. 1206, killed in 7 days by 200 units, showed some marrow necrosis, and shallow subendosteal Howship's lacunae, containing a few osteoclasts; while No. 1196 killed in 11 days by 500 units showed very extensive mar-

row necrosis, considerable subendosteal resorption, and fractures at some of the costochondral junctions. Some of these animals showed enlargement of the Haversian canals which contained connective tissue and osteoclasts in Howship's lacunae, and some also showed slight degrees of marrow fibrosis.

These experiments show that parathormone, given in separate doses over a period of several days, produces bone changes and kills adult guinea pigs which would not be affected by the same amount of the hormone given at a single injection. However, if care is taken not to

TABLE II

Dose	Duration	No. 1204 Weight	No. 1207 Weight
units	days	gm.	gm.
20	4	950	810
40	4		
0	2	900	750
20	2	900	750
40	2	900	780
60	1		
0	1	900	770
20	1		
40	1		
60	3	880	723
0	3	850	715
20	1	855	705
40	3	875	710
60	1	855	700
40	8	860	725
60	13	820	700
0	1	875	720
60	18	875	725

increase the dose too rapidly, or if the treatment is intermitted when the animals appear sick from hyperparathyroidism, the injections can be continued for a long time, with increasing dosage. Two adult guinea pigs, weighing 950 and 810 gm., respectively, received 3040 units of parathormone over a period of 69 days, as shown in Table II.

Guinea Pig 1207 was the younger of the two animals, and showed the greater number of changes. There was very extensive enlargement of the Haversian canals, which contained much young connective tissue. Some of the enlarged resorption spaces showed deep Howship's lacunae and numerous osteoclasts.

This animal also showed a slight amount of subperiosteal and subendosteal resorption, with some subendosteal fibrosis. However, in Guinea Pig 1204 considerable evidence of healing was encountered. There was practically no subperiosteal or subendosteal resorption, and the enlarged Haversian canals, although filled with young connective tissue, showed smooth surfaces, and lining of the surfaces by osteoblasts as another indication of the healing (Fig. 13).

An adult guinea pig, weighing 960 gm., received a total of 4020 units of parathormone in the same period. The dosage was the same as for Guinea Pigs 1204 and 1207 until the 52nd day, after which the following amounts were given daily: 60 units for 5 days; 80 units for 2 days; 100 units for 2 days; 120 units for 2 days; 140 units for 2 days; 160 units for 2 days; 180 units for 2 days; and 200 units for 1 day.

At autopsy this animal showed beaded ribs and bones that were definitely softened. Microscopical examination showed marked enlargement of the Haversian canals. So extensive was the resorption within these canals that in places the cortex was almost completely replaced by young connective tissue. Some of the long tubular bones and the ribs showed subendosteal resorption, with Howship's lacunae and osteoclasts.

These experiments show that the adult animal can tolerate great amounts of parathormone if the earlier injections are graded so as not to produce fatal hyperparathyroidism. The bone changes produced during a long course of parathormone injections in adults have a tendency to heal as the treatment is continued. However, when the dose of parathormone is further increased, as during the final 13 days in the guinea pig last discussed, indications of more acute hyperparathyroidism reappear, and manifest themselves by extensive subendosteal resorption.

SUMMARY AND DISCUSSION

These studies have shown that the bones of guinea pigs given daily injections of parathormone from the age of 2 to 7 days to the age of 110 to 120 days, show relatively very little effect after receiving 20 units daily during the last 65 to 87 days of treatment. But it is probable that their bones underwent decalcification early in the treatment and that subsequently the parathormone, continued at the same dosage, did not maintain the effects on the bones. Healing finally occurred despite it.

The bones of guinea pigs treated with intermittent injections of large doses of parathormone from the time they were 1 week old to

the age of 95 to 145 days also showed relatively few changes at the end of the treatment. The injections were given at intervals of 7 to 11 days, and were stepped up from 60 units to 140 units. From our previous experience (1) we infer that the earlier injections of parathormone produced very extensive bone changes which healed in the intervals between the injections. As the guinea pigs became older the injections of parathormone did not produce as severe effects.

We have found in our studies of experimental hyperparathyroidism that the bone changes after a single large dose of parathormone in young guinea pigs are soon healed. The study of a series of animals shows that healing begins at about the 48th hour after injection, and proceeds rapidly. Between the 8th and 14 days, callus may be observed at the costochondral junctions, where fractures had occurred. Now the endosteum may be lined by osteoblasts and the vessel canals by new formed bone.

In adult guinea pigs extremely large single doses had little effect on the bones in 48 hours, even though the dose killed the animal. It was only when three doses pyramided over a period of 48 hours and totaling 2580 units of parathormone were given, that moderately severe bone resorption could be demonstrated in the adult.

The elevation of serum calcium may be considered as one of the indices of calcium mobilization in experimental hyperparathyroidism. When the rate of calcium excretion exceeds the rate of its mobilization, or when the animal is on a low calcium diet, hypercalcemia may be absent. It is possible to raise the serum calcium of adult guinea pigs by large single doses of parathormone, but the resulting rise is not as great as in the young (2). This is confirmatory evidence of the fact that calcium is mobilized much less rapidly from the bones of old animals than from those of young ones. Collip pointed out that young normal dogs are more susceptible to parathormone (6). This observation was corroborated by Morgan and Garrison (7). We found that the same difference held also in experimental hyperparathyroidism produced in dogs by repeated doses of parathormone (8). In man, clinical experience likewise indicates the necessity of using relatively large doses of parathormone to raise the serum calcium of adults. The serum calcium of middle-aged or old adults does not rise significantly unless as much as 100 units or more of parathormone are

given daily for a number of days. Charts VI and VII, in a recent paper by Merritt and Bauer (9), support our findings of the relative difficulty of obtaining a significant elevation of serum calcium in adults.

If adult guinea pigs are given daily injections of parathormone which are rapidly stepped up, the animals may be killed by the ensuing acute hyperparathyroidism, only slight bone changes being produced. However, a careful avoidance of the induction of acute hyperparathyroidism by gradual stepping up of the parathormone dose permits the employment of doses continued over a long period of time that could not possibly have been tolerated otherwise. Furthermore, healing of the lesions thus produced may occur, in spite of the continuance of parathormone at this level.

It seems likely that the difference in response of young and old guinea pigs to single doses of parathormone, as indicated by the bone changes, as well as by the serum calcium and phosphorus, is related to the more rapid rate of mineral metabolism in the young, actively growing animals. The calcium mobilizing effect of parathormone is most prominent in actively growing young animals, the calcium being withdrawn from the most readily available stores—the regions of most active new bone formation and most active bone reconstruction (10).

In the adult animal the calcium reserves (in the formed bone) are less susceptible to the calcium mobilizing effect of parathormone. The adult guinea pig will show relatively slight bone changes even as a result of extremely large, fatal doses of parathormone. Repeated doses, as is well known, will produce, by pyramiding, greater effects than the entire amount administered at one time. In this type of experiment the young again show greater susceptibility of the bone than the adult. In time, however, some compensation takes place, and the effects of the same doses are decreased until finally healing may occur in spite of the continued treatment. Increase of the dose, however, again elicits the parathormone effects upon the bone, as well as upon the serum calcium and phosphorus, without toxic changes (1, 8). It would seem that some compensation sets in which may be overcome by increasing the dose. This compensation is especially evident in the experiments in which the parathormone doses were stepped up gradually from small amounts.

In addition to the compensation observed in young and adult animals as a result of repeated injections of parathormone, we must also consider the possibility that there is a compensating mechanism in adult animals more effective than in the young. That compensation occurs is unquestionable but its nature is not clear. Apparently it is less effective during pregnancy, doses of parathormone which produce only slight bone changes in ordinary adults causing very severe lesions in advanced pregnancy (11).

Parathormone has been shown to produce only one primary effect on bone, and that is decalcification. This may come about as the result of a change in the circulating tissue fluids, the salts being dissolved out of the organic matrix, and the latter disappearing secondarily. The process is most rapid in the vicinity of most active bone formation. The osteoblasts disappear from the surfaces of bone where dissolution is occurring, and at the same time the marrow connective tissue proliferates. Fusion of cells produces osteoclasts (12), which then proceed to remove the decalcified organic matrix, with the production of the deep lacunae of Howship. Frequently leucocytes are also observed actively phagocyting the decalcified organic matrix, and often leucocytes are observed within the osteoclasts (12). Healing is associated with the complete reversal of the process. The osteoclasts disappear, the connective tissue diminishes, osteoblasts reappear, and bone formation is resumed.

As we have previously stated (13), parathormone produces a more continuous effect than experimental acidosis and greater changes than are seen in experimental osteoporosis. A pronounced decalcification results from it which, with its sequelae, simulates von Recklinghausen's disease. The emphasis which the older pathologists laid on osteoclasts as a special feature of ostitis fibrosa cystica is justified, for in the experimental condition the appearance of great numbers of osteoclasts is a constant feature, whenever decalcification occurs (13).

There seems to be no doubt that the giant cell tumors found in ostitis fibrosa cystica are expressions of the same pathological response. The other features of the bone changes of hyperparathyroidism— marrow hemorrhage, cysts, fractures, and osteoid proliferation—are secondary to the primary decalcification. Progress of the pathological changes leads to circulatory stasis and cyst formation. Stresses and

strains exerted on the progressively weakening bone may result in microscopical or gross fractures. Osteoid tissue is, as we have previously pointed out (13), merely reparative in nature, being laid down as support to the weakened or fractured bone, or as a part of healing. Osteoid borders appear on bone surfaces 48 hours after one large dose of parathormone.

The mosaic picture which we have observed in the bones of some of our animals is produced by short and irregularly disposed cement lines resulting from rapid bone transformation. Schmorl (14) recently emphasized the mosaic-like appearance of the newly formed lamellar bone in Paget's disease (ostitis fibrosa deformans). The mosaic-like appearance of bone has also been described in local bone conditions, as *e.g.* syphilitic periostitis, and in bone in the vicinity of cysts and giant cell tumors in von Recklinghausen's disease (ostitis fibrosa cystica). However, Schmorl claims that in no disease is the mosaic appearance so constant and the arrangement of the cement lines so irregular as in Paget's disease. In chronic experimental hyperparathyroidism (von Recklinghausen's disease), the mosaic structure is not a prominent feature because of the progressive decalcification. But the bones of our young guinea pigs which received intermittent injections showed a mosaic-like appearance indicative of the periodic decalcifications and restorations which they had undergone.

CONCLUSIONS

1. Young guinea pigs are more susceptible than adult guinea pigs to the effects of single or repeated doses of parathormone, as shown by the bone changes.

2. Several successive daily doses of parathormone, in rapidly increasing amount, result in an accentuation of the effects.

3. In young and adult guinea pigs a compensation is established during prolonged parathormone treatment, which enables them to tolerate repeated large doses and which permits considerable repair of bone lesions produced earlier in the treatment.

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EXPLANATION OF PLATES

PLATE 7

FIG. 1. Cortex of the shaft of rib of a guinea pig given 20 units of parathormone for 87 days after some preliminary treatment with smaller doses. The animal weighed 269 gm. when the injection of 20 units was begun. Note that the bone is compact and no subperiosteal resorption is present. Compare with Fig. 2. Magnification 80 ×. Stained with hematoxylin and eosin.

FIG. 2. Cortex of the shaft of rib of a guinea pig given 10 units of parathormone for 16 days, shown for comparison with Fig. 1. The animal weighed 260 gm. when treatment was begun. The cortex is thin; there is marked subperiosteal resorption; the vessel canals are enlarged and filled with cellular connective tissue; the marrow is congested. Magnification 65 ×. Stained with hematoxylin and eosin.

FIG. 3. The costochondral junction of a rib of a guinea pig injected daily for 16 days with small, gradually increasing doses of parathormone. This was followed by intermittent injections begun when the animal was 1 month old, and weighing 272 gm. These were given at intervals of 7 to 11 days, and stepped up from 60 to 140 units. Note fractures of the cortex and the scarifying of the marrow. The only severe lesions in this animal were observed at the costochondral junctions. Magnification 65 ×. Stained with hematoxylin and eosin.

FIG. 4. The cortex of the shaft of the rib shown in Fig. 3. Note the compactness of the bone and the absence of subperiosteal and subendosteal resorption. Magnification 80 ×. Stained with hematoxylin and eosin.

FIG. 5. The epiphysis and metaphysis of the upper end of the tibia of the same guinea pig as in Fig. 3. Note the complete absence of resorptive changes in the metaphysis. Magnification 80 ×. Stained with hematoxylin and eosin.

PLATE 8

FIG. 6. The skull of the guinea pig described in Fig. 3 legend. Note the numerous cement lines which give the bone a mosaic appearance. These lines show the effect on the bone of the intermittent parathormone injections. Magnification 125 ×. Stained with hematoxylin and eosin.

FIG. 7. The costochondral junction of a rib of a young guinea pig weighing 300 gm., shown for comparison with Figs. 3 and 5. The animal was killed 48 hours after the injection of 60 units of parathormone. The marrow is degenerated; the cortex is fractured in numerous places; the columns of endochondral bone are splintered. Magnification 65 ×. Stained with hematoxylin and eosin.

FIG. 8. Cortex of the shaft of the rib shown in Fig. 7. The lymphoid marrow has disappeared, and the marrow cavity is filled with blood, the result of free hemorrhage. The cortex shows extensive resorption and enlargement of the vessel canals, which contain connective tissue. Compare with Fig. 4. Magnification 65 ×. Stained with hematoxylin and eosin.

FIG. 9. The costochondral junction of the rib of an adult guinea pig weighing 1040 gm., injected with 2080 units of parathormone. The animal died 40 hours later showing no changes in the bone, but some congestion of the marrow. Magnification 85 ×. Stained with hematoxylin and eosin.

PLATE 9

FIG. 10. The cortex of the shaft of the rib shown in Fig. 9. Note the complete absence of subperiosteal and subendosteal resorption. The bone is compact. Magnification 85 ×. Stained with hematoxylin and eosin.

FIG. 11. The cortex of the shaft of a rib of an adult guinea pig injected with 2580 units of parathormone in 48 hours. About 48 hours after the last injection the animal was killed. Only with such large, pyramided doses was it possible to induce subendosteal resorption. Magnification 85 ×. Stained with hematoxylin and eosin.

FIG. 12. Part of the epiphysis and metaphysis of the lower end of the femur of the animal described in the legend of Fig. 11. Note evidences of resorption of the trabeculae of the epiphysis and metaphysis. Magnification 85 ×. Stained with hematoxylin and eosin.

FIG. 13. Cortex of the shaft at the middle of the femur of an adult guinea pig receiving 3040 units of parathormone over a period of 69 days. Care had been taken not to increase the dose too rapidly during the early critical periods. Note the marked enlargement of the vessel canals which are filled with cellular connective tissue and contain osteoclasts in Howship's lacunae. Magnification 40 ×. Stained with hematoxylin and eosin.

PLATE 7

PLATE 8

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PLATE 7

PLATE 8

(Jaffe *et al.*: Bone lesions in hyperparathyroidism)

THE ARTHUS PHENOMENON

LOCAL ANAPHYLACTIC INFLAMMATION IN THE RABBIT PERICARDIUM, HEART, AND AORTA

By DAVID SEEGAL, M.D., BEATRICE CARRIER SEEGAL, M.D., AND
ELIZABETH L. JOST

(From the Departments of Medicine and Bacteriology, College of Physicians and
Surgeons, Columbia University, and The Presbyterian Hospital, New York)

PLATES 10 AND 11

The Arthus phenomenon has been studied most extensively in sub-cutaneous tissue but has also been produced in certain viscera. Unpublished work[1] on the routes of absorption of diffusible and non-diffusible substances from the rabbit and dog pericardial sac led us to believe that this might be a site which was especially suitable for the study of the Arthus phenomenon. The advantage in using this closed space is due to the ease with which antigen can be applied to sensitized tissue without significant trauma. The present paper reports the results of such experiments upon the rabbit pericardium, heart, and aorta.

After repeated parenteral injections of a foreign protein into rabbits Arthus (1) observed that a subsequent subcutaneous injection of the same protein resulted in a local, severe, sterile, inflammatory reaction. If the protein was injected into a normal untreated animal in much greater quantities it produced no demonstrable effect. This destructive lesion produced in a sensitized animal by the subcutane-ous injection of the specific antigen has since been known as the "Arthus phe-nomenon." Gerlach (2) has given a careful comparative histological description of this sequence of events in guinea pigs, rabbits, rats, dogs, and in man. By excising, in the sensitized animals, a portion of the subcutaneous tissue at different intervals after reinjection of the specific antigen he could accurately trace the different stages of the inflammatory process. As early as 1 hour after inoculation,

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[1] This work was carried out in the Department of Pathology, Harvard Medical School, while one of us (D. S.) held the James Jackson Cabot Student Fellowship.

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swelling of the connective tissue and edema with compression of the vessels and disappearance of the capillaries was evident. This was followed by a leucocytic infiltration, hemorrhage, and necrosis of the involved tissue, leading eventually to the formation of a sterile abscess. At the end of 8 days, new connective tissue was found at the periphery and healing proceeded with the formation of scar tissue covered by a hairless epidermis. In contrast to this, control animals which had not previously been sensitized, showed only a slight infiltration of leucocytes at the site of injection, lasting about 24 hours. Certain immunological studies, notably those of Opie (3), have demonstrated that the union of antigen and antibody in or on the fixed tissue cells is necessary for the appearance of the Arthus phenomenon.

Several investigators have shown that this local anaphylactic inflammation is not peculiar to the subcutaneous tissues, but can occur in other tissues of the body, as for example, the viscera and the endothelial cavities. It has been produced in the lungs, Busson (4); von Friedberger (5); Ishioka (6); Schlecht and Schwenker (7); and Opie (8); in the testicle, Long and Seyfarth (9); in the joints, Landouzy, *et al.* (10); Schlecht and Schwenker (7); and Klinge (11); and in the peritoneum, Longcope (12); Roessle (13). Shapiro and Ivy (14) have produced gastric ulcers by injection of the homologous antigen into the gastric submucosa of sensitized rabbits. Hepler and Simonds (15) have studied the inflammation which resulted when the specific antigen was injected into the kidney parenchyma of a sensitized rabbit. Such injections produced hemorrhage, necrosis of the tubules, and a leucocytic exudate while similar injections of the antigen into normal rabbits' kidneys produced hemorrhage only. Alessio (16) and Choi (17) have reported similar necrosis and leucocytic exudate in the livers of immune rabbits following the direct injection of the specific antigen into this organ. The findings of Coulter and Pappenheimer (18) and of Long and Finner (19) although accomplished by a different technique are nevertheless significant in this connection. Coulter and Pappenheimer obtained injury to the glomeruli of rabbits' kidneys by injecting either bacterial products or egg white into the renal artery of animals that had been sensitized by previous parenteral injections of the specific antigen. Long and Finner produced a glomerulonephritis in tuberculous swine by the injection of tuberculin into the renal artery. Our own experience with the occurrence of anaphylactic inflammation in the kidney and liver of sensitized rabbits after direct introduction of the antigen into these organs agrees with the observations of Hepler and Simonds, Alessio and Choi.

EXPERIMENTAL DATA

A group of seventeen rabbits received daily subcutaneous injections of 1 cc. of a 20 per cent solution of fresh egg white. After the sixth to the tenth injection, most of these animals developed a characteristic local inflammation similar to that described by Arthus. They were then allowed to rest from 4 to 5 days, at which time their precipitin titer against egg white ranged from 1:20,000 to 1:160,000.

At this time 1 cc. of a 20 per cent solution of fresh egg white was injected into the pericardial sac of each of these animals in the following manner: Under ether anesthesia a parasternal thorocotomy was performed and upward traction was exerted on the medial margin of the wound. This procedure stretched the pericardium so that the direct injection into its sac could be accomplished with accuracy and without injury to the myocardium. Artificial respiration was unnecessary since the rabbit mediastinum is complete. A control series of fifteen normal rabbits, whose sera contained no precipitins against egg white, received similar intrapericardial injections of the egg white solution. The sensitized animals with their controls were sacrificed at intervals from 2 to 18 days after the operation by the intravenous injection of 5 to 8 cc. of 95 per cent alcohol. Four sensitized animals died within the first 4 days. Routine smears and cultures were taken from all the pericardial sacs and the tissues were studied histologically after Zenker fixation.

Sensitized animals sacrificed 2 to 4 days after the intrapericardial injection showed a characteristic gross picture. On resection of the anterior chest wall, the heart appeared dilated and its surface was colored a deep purplish red. The parietal pericardium was slightly thickened and in a few instances was lightly adherent to the epicardium. In about one-fourth of the seventeen experimental animals the pericardial fluid was serosanguineous in character, increased in amount, and contained free bits of fibrin. The epicardium in such instances was deeply congested and on its violaceous surface a finely granular deposit was present. This fibrinous epicarditis was frequently present even when there were no free masses of fibrin in the pericardial fluid. In addition to the deep congestion of the outer aspect of the heart there were also many tiny fresh subepicardial hemorrhages scattered between the dull, stippled, grey areas. These hemorrhagic areas were frequently present in the adventitia of the intrapericardial portion of the great vessels. A striking edema in the loose supracardiac tissue about the thymus gland was sometimes present. On opening such hearts the chambers appeared markedly dilated and occasionally petechial hemorrhages were apparent on the cut surface of the muscle. A picture of the heart of an experimental animal as compared with a control is shown in Fig. 1.

These obvious gross changes were present in thirteen of the seventeen sensitized animals (76 per cent). Of the fifteen controls no gross changes were noted following the injection of the egg white solution into the pericardial cavity.

Microscopic studies were made of many sections of tissue selected from the pericardial sac, the four chambers of the heart and the intrapericardial and extrapericardial portions of the great vessels. Fifteen of the seventeen experimental animals (88 per cent) showed characteristic microscopic lesions in some portions of the heart muscle and pericardium. Changes in the myocardium occurred more frequently in the auricles than in the ventricles but in the more striking instances the entire heart muscle participated in the process. In half of the animals there were changes in the adventitia of the aorta and in three there was a peculiar lesion involving the intima. The characteristic pathology in the heart muscle consisted of focal necroses involving muscle cells, connective tissue, and blood vessels. In these areas of necrosis there was striking hemorrhage and a cellular infiltration made up chiefly of polymorphonuclear leucocytes in the animals sacrificed as early as 2 days after the intrapericardial injection. In the animals sacrificed at 4 days the polymorphonuclear cells were largely replaced by lymphocytes and large mononuclear cells. Frequently, these cells were found assuming a perivascular arrangement. These changes are shown in Fig. 2.

The lesions of "spontaneous interstitial myocarditis" described by Miller (20) could be found after careful study in about half of these animals. In most cases there was no difficulty in distinguishing the hemorrhagic necrotic areas from the "spontaneous lesions" which consist of interstitial collections of lymphocytes, endothelial cells, and fibroblasts. When the inflammatory changes in the sensitized animals were marked, it was impossible to determine whether they were superimposed upon a "spontaneous" lesion.

The pathology in the aortae was confined to the adventitia in most instances and consisted of edema, hemorrhage, a peculiar hyalinoid deposition, and a cellular infiltration comparable to that in the myocardium. This picture is shown in Fig. 3. In three aortae there were changes in the intima consisting chiefly, in two instances, of a subendothelial collection of polymorphonuclear cells unaccompanied by cholesterin collections or other evidence of spontaneous arteriosclerosis. In the third animal the changes in the intrapericardial aorta were striking. Here there was complete destruction of the intima and a dense polymorphonuclear leucocytic infiltration invaded

the inner half of the blood vessel. Portions of this necrotic area were covered by an early thrombus. Gram stains of this area failed to disclose the presence of any organisms.

Comparable microscopic sections of the control animals showed no such lesions. In none of the sections was there any evidence of pericarditis, adventitial or intimal changes in the great vessels, or the hemorrhagic focal necroses found in the myocardium of the sensitized group. In 55 per cent of this control group the characteristic lesions of "spontaneous interstitial myocarditis" were present. They could not be confused with the type of histopathology present in the sensitized animals. The routine direct smears and cultures of the pericardial fluids were uniformly negative except for a culture of *Staphylococcus aureus* obtained from one of the control animals. However, there was no microscopic change demonstrable in the sections of this animal.

Of the four sensitized animals which died during the first few days after the operation, a rabbit which was found dead $1\frac{1}{2}$ days after injection had the most striking cardiac pathology of any animal in the series and may have died as a result of this lesion, the only other contributing factor being a marked liver coccidiosis. Another rabbit is noteworthy which died 2 days after the intrapericardial injection. At autopsy the heart was greatly dilated and its epicardial surface was studded with hemorrhages. The liver was of a deep purple color and appeared congested. There was about 100 cc. of straw-colored ascitic fluid. Although there were some old scars of coccidiosis infection in the liver no cysts were found in the peritoneal cavity. Microscopically, the heart showed a striking myocarditis and it would appear that the cause of death in this instance was secondary to myocardial insufficiency with failure of the right portion of the heart.

Of two sensitized rabbits sacrificed at later intervals, one rabbit killed on the 8th day after the operation showed no demonstrable lesions but an animal sacrificed 18 days later had a definite though slight myocarditis.

Although no absolute correlation was found between the height of the precipitin titer, the subcutaneous Arthus phenomenon and the subsequent cardiac lesion, the protocols favor the impression that when the precipitin titer is high and the subcutaneous Arthus phe-

nomenon is marked, there will be a more pronounced lesion in the pericardium, heart, and aorta. The converse of this is not always true. This finding agrees with our previous observations on the relation between the height of the precipitin titer in sensitized rabbits and the extent of liver and kidney lesions after direct introduction of the antigen into the particular organs.

DISCUSSION

An intense inflammatory reaction in the pericardium, heart, and intrapericardial aorta can regularly be produced in a sensitized rabbit by the intrapericardial injection of the homologous antigen. The pathology described in this paper may be considered to be of an allergic nature. It is apparent that the appropriate union of antigen and antibody in such an area as the pericardial cavity may lead to striking changes. The lesions are not confined to the pericardial sac since a striking myocarditis is present in most of these animals.

A possible explanation for the development of myocarditis in sensitized animals subjected only to an intrapericardial injection of the antigen may be found in previous work in which we had shown that such a substance as trypan blue would extend back through the rabbit heart after intrapericardial injection. At the end of 15 minutes the endothelial cells between the muscle fibres would be deeply stained with the dye, whereas none of the pigment could be found in the liver and spleen. The trypan blue also deeply stained the great vessels around the base of the heart. Particulate matter such as carmine when injected into either the rabbit or the dog pericardial sac did not penetrate the deeper portions of the cardiac wall, probably on account of the larger size of the carmine particles. Most of this pigment was found in macrophages in the subepicardial zone and in the dilated lymph channels and enlarged lymph glands of the anterior, posterior, and superior mediastinum. It is reasonable to suppose that the diffusible antigen in our present experiments might pass through the myocardium and produce the changes noted in the heart.

Myocardial lesions following repeated intravenous injections of foreign protein have been described by Longcope (21), Boughton (22), and Klinge (11). Longcope found extensive focal degeneration in the cells of the heart muscle following the repeated intravenous

injection of egg white or horse serum in rabbits. These areas were characterized by dense infiltration of small round cells which later gave way to scar formation. However, he found similar changes in 25 per cent of twenty-two animals which had received only one to three intravenous injections of the same foreign protein. He fails to report any pericarditis or aortitis in his series of animals. None of his protocols mention any hemorrhage or striking gross changes in the hearts. Boughton reported degeneration and regeneration of the endothelium of the smaller arteries of the hearts of guinea pigs which he had repeatedly shocked with egg white or beef serum. He described similar findings in the blood vessels of the liver, spleen, and kidney of his animals. Klinge repeatedly injected horse serum into the joints and skin of rabbits. The hearts of such animals showed small focal necroses and areas of perivascular infiltration of round cells. The photographs of these lesions closely resemble those of "spontaneous interstitial myocarditis" described by Miller. Myocardial lesions following anaphylactic shock in the guinea pig have been reported by Gay and Southard (23). They described hemorrhages into the myocardium and also demonstrated fatty changes both in the muscle cells and in the endothelial cells of the capillaries in this organ.

CONCLUSION

An intense inflammatory reaction in the pericardium, heart, and intrapericardial aorta can regularly be produced in a sensitized rabbit by the intrapericardial injection of the homologous antigen.

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EXPLANATION OF PLATES

PLATE 10

FIG. 1. The hearts of two rabbits sacrificed 4 days after the intrapericardial injection of 1 cc. of a 20 per cent solution of fresh egg white. The figure on the left is that from an egg white-sensitized animal; that on the right is a normal control. These animals were approximately of the same weight. In the heart of the experimental animal note the dilated right ventricle and auricle and the deposition of large masses of fibrin over the dull epicardial surface. The injection and hemorrhages of the epicardial surface are evident in the drawing. ×1.

PLATE 11

FIG. 2. A section of the right auricle of a sensitized animal dying 2 days after the intrapericardial injection of 1 cc. of a 20 per cent solution of fresh egg white. Note the loss of striations in the muscle cells, the hemorrhage between these pale fibres, and the infiltration of polymorphonuclear leucocytes and round cells. Hematoxylin and eosin. ×300.

FIG. 3. The intrapericardial aorta of a sensitized rabbit sacrificed 4 days after the intrapericardial injection of 1 cc. of a 20 per cent solution of fresh egg white. Note in the adventitia the intense hemorrhage, and the cellular infiltration of leucocytes. Hematoxylin and eosin. ×300.
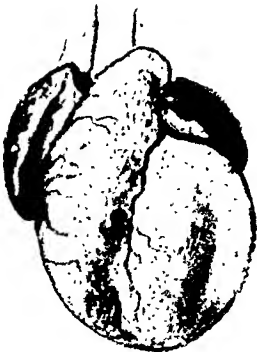
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(Seegal *et al.*: Arthus phenomenon)

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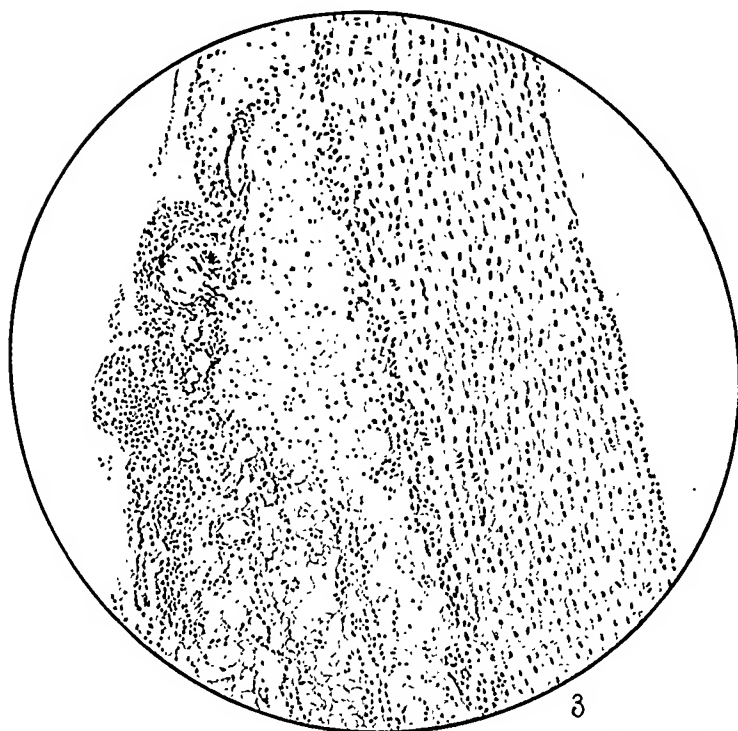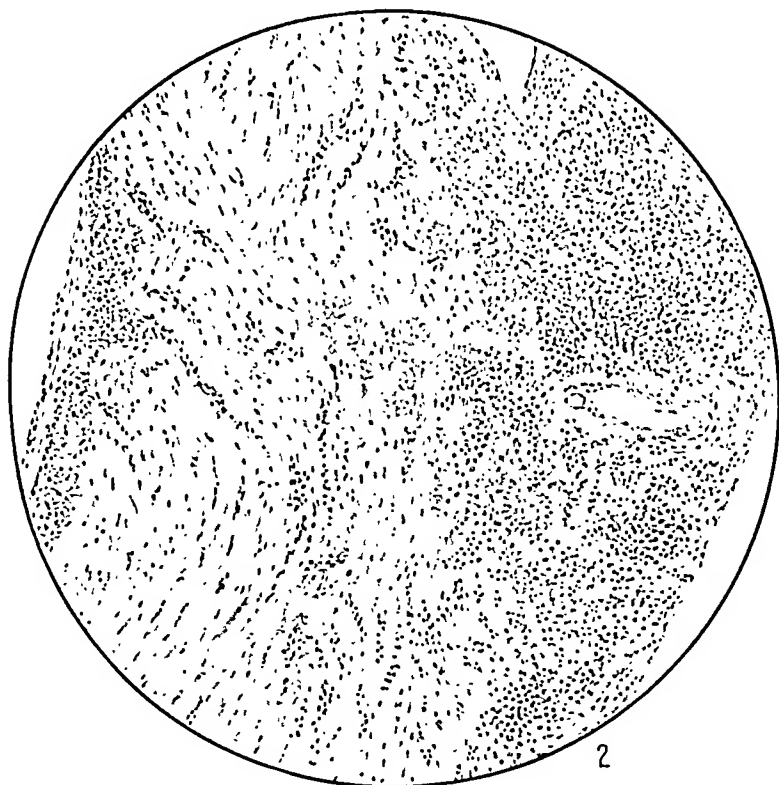
brain. Two rabbits died 7 days after the last injection of causes apparently unrelated to the experiment. Five were etherized at intervals of from 2 to 11 days. The brains were examined histologically. All but one animal, sacrificed at the 48 hour interval, showed changes characteristic of local allergic inflammation to be described presently.

None of the control animals died as a result of the experimentation. They were etherized and the brains were likewise studied histologically. While slight hemorrhages were present at the site of injection in one or two instances, none of the controls showed lesions comparable to those found in the sensitized series.

One factor in Group I appeared to us insufficiently controlled, namely that of trauma, adhesions, and mechanical rupture of large blood vessels as a result of repeated injections into the same area of the brain. We undertook, therefore, another experiment in which only one cerebral injection was to be done on each animal.

Group II.—This group consisted of ten sensitized animals and eight controls. The latter remained uninjected until the cerebral inoculation. The former were prepared by daily subcutaneous injections of 0.2 cc. of horse serum for 10 consecutive days. Most of the animals showed varying degrees of anaphylactic inflammation in the skin. 11 days after the last injection, the sensitized and control animals were given 0.2 cc. of horse serum into the left cerebrum.

Although no untoward effects were observed immediately, one sensitized animal was found dead and another dying 8 hours after the cerebral injection, the others survived symptomless. Examination of the brains of the two rabbits showed the typical sterile inflammatory changes characteristic of local anaphylactic reaction. The control group never showed any untoward symptoms as a result of this injection. On histological examination two of the control brains presented a slight hemorrhage along the needle tract. This was not associated with any of the other changes seen in the Arthus phenomenon and was due undoubtedly to mechanical trauma.

Group III.—The last group was treated similarly to Group II, except that a different antigen was used. Ten rabbits were sensitized with egg albumen, and 12 days after sensitization, they, together with seven normal controls, were each given 0.2 cc. of egg albumen into the left cerebrum.

Among the sensitized animals in this group three responded with tonic and clonic muscular contractions 1 minute after the cerebral injection, following which they kept rotating toward the right for 15 to 30 minutes.   One of these animals died $\frac{1}{2}$ hour after the attack. A fourth showed the rotary movements without preceding convulsions, a fifth one exhibited similar signs the next day to a slight degree.   The four surviving rabbits which had shown clinical symptoms were etherized 3, 8, and 17 days later.   As in the previous experiment, the controls showed no response whatsoever to the injection, but were etherized for histological comparison with the sensitized animals.   Of the five experimental animals that had shown symptoms of cerebral disorder, four had histological changes characteristic of local anaphylactic inflammation in the brain.   The fifth showed no pathological changes in the brain, but since the reaction in this case was mild, and the autopsy was not performed until 8 days after the injection, the signs of inflammation may conceivably have disappeared by this time.

The additional data obtained from these two groups made our conclusions certain that, while individual animals may differ in the degree of sensitivity, in most rabbits, given the proper experimental conditions, a localized anaphylactic inflammation of the brain can be made to develop.

Pathological Findings

In the gross the lesion seen in the brains of the sensitized animals is very striking (see Fig. 1).   The side of the brain containing it is congested and enlarged and the midline structures are displaced toward the opposite side.   On transverse section the entire center of the hemisphere is softened and hemorrhagic and the narrow margin of approximately normal tissue is edematous.   The appearance suggests the results of a miniature explosion which had taken place in the middle of that hemisphere.

The histological picture (Fig. 2) of the Arthus reaction in the brain resembles very closely that described in the skin and subcutaneous tissue by Arthus and Breton, Gerlach, Doerr, and others, allowing for the differences in the fundamental structure of these tissues.

In the rabbit's brain 24 hours after the injection, the lesion often involves the entire hemisphere on the side of the injection and may

SUMMARY AND CONCLUSIONS

1. Sixteen out of seventeen rabbits actively sensitized to various antigens by repeated cerebral and intravenous injections showed upon intracerebral reinjection of the same antigen local anaphylactic inflammation of the brain at the site of inoculation.

2. Six out of twenty rabbits actively sensitized to either horse serum or egg albumen by extracerebral injections, showed, upon introduction of the homologous antigen into the cerebrum, local anaphylactic inflammation at the site of inoculation.

3. The pathological picture of the Arthus phenomenon in the brain of the rabbit resembled that seen in the skin, after allowing for differences in the fundamental structure of the tissues involved.

4. None of the control animals exhibited lesions comparable to those found in the experimental animals. A few controls showed slight hemorrhages due to mechanical injury of blood vessels.

5. Clinical symptoms of varying degrees of severity, often leading to the death of the rabbit were observed in the sensitized animals. These symptoms were referable to the site of injection.

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EXPLANATION OF PLATE 12

FIG. 1. A cross-section of the brain of a sensitized rabbit 24 hours after the intracerebral injection of the specific antigen, horse serum. Note the extensive hemorrhage and edema pushing the structures of the midline over to the opposite side. Drawing ×2 natural size.

FIG. 2. An area from the brain of a sensitized rabbit 24 hours after the intracerebral injection of the specific antigen, horse serum. Note the marked hemorrhage, extravasation of leucocytes, and edema of the cerebral tissue. Hematoxylin-eosin stain. Magnification ×300.

FIG. 3. An area from the brain of a control rabbit 24 hours after the injection of horse serum. Note the slight hemorrhage incident to mechanical trauma to a small blood vessel. Extravasation of leucocytes and edema are absent. Hematoxylin-eosin stain. Magnification ×300.

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(Davidoff *et al.*: Arthus phenomenon)

SUMMARY AND CONCLUSIONS

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5. Clinical symptoms of varying degrees of severity, often leading to the death of the rabbit were observed in the sensitized animals. These symptoms were referable to the site of injection.

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EXPLANATION OF PLATE 12

Fig. 1. A cross-section of the brain of a sensitized rabbit 24 hours after the intracerebral injection of the specific antigen, horse serum. Note the extensive hemorrhage and edema pushing the structures of the midline over to the opposite side. Drawing ×2 natural size.

Fig. 2. An area from the brain of a sensitized rabbit 24 hours after the intracerebral injection of the specific antigen, horse serum. Note the marked hemorrhage, extravasation of leucocytes, and edema of the cerebral tissue. Hematoxylin-eosin stain. Magnification ×300.

Fig. 3. An area from the brain of a control rabbit 24 hours after the injection of horse serum. Note the slight hemorrhage incident to mechanical trauma to a small blood vessel. Extravasation of leucocytes and edema are absent. Hematoxylin-eosin stain. Magnification ×300.

PLATE 12

2

1

3

(Davidoff *et al.*: Arthus phenomenon)

IMMUNOLOGICAL REACTIONS OF PNEUMONIC PLEURAL FLUIDS*

By MAXWELL FINLAND, M.D.

(From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston)

A study of the pathological and immunological changes that take place in the vicinity of an area of local inflammation may shed some light on the general protective mechanism of the body against infection. During the course of lobar pneumonia the pleura, being contiguous to the lung and sharing, at least in part, in its inflammatory reactions, offers an opportunity for such a study. Furthermore, with the recognition of the value of specific antipneumococcic serum in the treatment of certain types of lobar pneumonia, the problem of whether or not such serum reaches the lung and pleura becomes of practical importance. The present work consists, primarily, of the application of common bacteriological and immunological methods to a study of pleural fluids from patients with pneumococcic pneumonia. The cytological features of such fluids as revealed by observations with a supravital technique are included in a separate communication (1). It was hoped that some correlation between the immunological reactions and the fate of the infectious process might be found.

LITERATURE

Since Andral (2) and Laennec (3) first demonstrated clinically the existence of pleural effusions in lobar pneumonia, much has been written, especially in French literature, on this subject. Netter (4), however, published the first extensive study of the bacteriology of purulent pleural effusions and emphasized the frequency of the pneumococcus as an etiological agent. He showed that each kind of organism imparted to the fluid characteristics which were specific for the par-

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ticular organism involved. From 6 of the 109 fluids that this author studied, no organisms could be recovered either by cultural methods or by animal inoculations, using mice, rabbits, and guinea pigs. Pneumococci had previously been cultured from purulent pleural exudates by Friedländer (5), Netter (6), and Fränkel (7). More recently, Locke (8) collected the bacteriological findings in 478 cases of acute empyema, differentiating the various types among the pneumococci. The frequency of the pneumococcic etiology and the large predominance of Type I among the various types was clearly demonstrated.

Siems (9) quoted a case from Griffon (10) in which agglutination of pneumococci was demonstrated in a pneumonic pleural fluid by growing the organisms in it. Siems believed that the pneumococcic etiology of postpneumonic effusions could be demonstrated not only by culture but also by serological methods applied to the blood serum and to the fluid. In one instance he demonstrated that a pleural fluid had the power to agglutinate the patient's own strain of pneumococcus when it failed to agglutinate the laboratory strain. Cole (11) showed that empyema fluids contained no protective substances or agglutinins but that they inhibited the protective properties of antipneumococcic sera specifically. Floyd (12) obtained "positive precipitin tests" with empyema fluids and noted phagocytosis of pneumococci by the cells in these fluids.

Duyck (13) found fluid containing pneumococci in the pleural cavity of almost every one of his cases during the acute stage of lobar pneumonia, especially in the presence of septicemia. In the latter type of case he found large numbers of organisms in the fluid. In spite of this fact, these fluids, when injected fresh, not only failed to kill mice but were capable of protecting them against lethal doses of pneumococci. This protection he attributed to leucocytic ferments. He treated cases by removing this fluid and injecting it into the same patient subcutaneously. Curphy and Baruch (14) have utilized a similar principle in immunizing horses for the production of antipneumococcic sera.

Pepper (15) demonstrated agglutinins for *B. typhosus* in a pleural effusion accompanying typhoid fever. The agglutinin titer in the fluid was 1:800 and in the blood serum 1:1000. Courmont (quoted by LeDamany (16)) demonstrated agglutination of tubercle bacilli by pleural fluid, but the method was thought by LeDamany not to be infallible. Many other authors have demonstrated antigen and antibodies in tuberculous pleural effusions (17).

General immunological characteristics of exudates and transudates have been studied by a number of workers, some of whom examined pneumonic pleural fluids. Hemolysins and complement have been found in exudates but not in transudates (18), and the cell-free exudates have been shown to be bactericidal for many organisms, including the pneumococcus (19).

Numerous workers have made studies of absorption from the pleural cavity. The literature, however, on the passage of immune substances from the blood into the pleural cavity or other closed cavities is scant. The most significant observations have been made on the cerebrospinal fluid in animals. Mott (20) found that

most drugs and tetanus toxin do not pass from the blood into the cerebrospinal fluid, whereas materials injected into the spinal canal appear rapidly in the blood. Flexner and his coworkers (21) were unable to detect neutralizing substances for the virus of poliomyelitis in the cerebrospinal fluid of actively immunized monkeys. They also failed to demonstrate agglutinins for meningococci in the fluid of actively or passively immunized normal rabbits. If, however, they produced an aseptic meningitis by injecting saline or normal horse serum into the subarachnoid space, they could demonstrate these antibodies. Freund (22) found that the cerebrospinal fluid of animals immunized with killed typhoid bacilli contains agglutinins for these organisms but the titer is very low compared to that of the blood serum. Normal animals passively immunized by the intravenous injection of typhoid immune serum were also shown to have typhoid agglutinins in their cerebrospinal fluid after a lapse of several hours. He explained the negative results previously mentioned by the low titer of antibody in the fluid.

Menkin reviewed the literature on the dissemination of substances from the site of injection (23) and showed that foreign protein introduced in the circulating blood of a rabbit accumulates in an inflamed area in greater concentration than in normal tissues (24).

Materials and Methods

31 pleural fluids from 19 patients with typical lobar pneumonia were studied, and 4 fluids from 3 patients with other diseases were studied for controls. A pneumococcus was obtained in each of the former cases from the sputum or blood culture or both. Type I pneumococci were obtained in 10 cases, Type II in 5 cases, Type III in 3 cases, and Type XVIII in 1 case.[1] Felton's bivalent (Types I and II) concentrated antibody solution was used in the treatment of 5 Type I and 3 Type II cases. 3 patients were treated with sera prepared by the Felton method from normal horse serum (Case 7) and from antimeningococcus serum (Cases 16 and 17). In 1 instance (Case 18) there was an opportunity to study fluid both before and after serum treatment.[2] All of the therapeutic sera were administered intravenously.

The fluids were secured, under aseptic precautions, by thoracentesis, choosing a point in the area of maximum percussion dulness. A solution of 1 or 2 per cent novocaine was injected through a 24 gauge needle to anesthetize the skin and underlying tissues, including the parietal pleura, a total of 1 or 2 cc. being used in the process. The fluid was then aspirated into a dry syringe through an 18 or 20

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[1] All of the sera used in typing were obtained through the courtesy of Dr. William H. Park, Laboratories of the New York City Department of Health.

[2] The therapeutic sera were prepared and supplied through the courtesy of Dr. L. D. Felton, Department of Preventive Medicine and Hygiene, Harvard Medical School, and of Dr. Benjamin White, Antitoxin and Vaccine Laboratory, Massachusetts State Department of Public Health.

gauge needle. Some of the fresh fluid was then inoculated into rabbit's blood broth and streaked on an agar plate containing rabbit's blood, and 1 cc. was injected into a mouse for routine pneumococcus typing. The remainder was allowed to clot, then centrifuged and the supernatant fluid stored in the ice box for later studies. In most instances, venous blood for culture and for the determination of circulating antibodies was obtained at about the time of the thoracic puncture.

The hydrogen ion concentration was obtained with the use of indicators by comparison with standard buffer solutions as recommended by Clark (25).

Antibody Determinations.—The *protection* titer of the fluids was determined by a mouse technique similar to that employed by Dochez (26). *Agglutinins* for pneumococci were shown by a technique similar to that employed by Tillett and Francis (27). The meningococcic antigen used in 2 instances was a formalized saline suspension of 1 of the strains employed in the preparation of the therapeutic sera. The agglutinations of this antigen were carried out by heating at 55°C. for 12 to 18 hours. *Precipitinogens* were demonstrated by adding 0.2 cc. of undiluted antipneumococcic typing sera[3] to equal amounts of the fluids, incubating for 2 hours at 37°C. and reading after storage overnight in an ice box. *Precipitins* were demonstrated in a similar manner using 1:1000 solutions of the specific polysaccharides of Types I, II, and III pneumococci. No attempt was made to obtain the exact titer of precipitin and precipitinogen. The presence of *horse serum* was detected in a similar manner by the precipitin reaction except that the anti-horse rabbit serum[4] was used in 1:2 dilution and varying dilutions of the fluids and sera from the patients were used in order to obtain quantitative results. The anti-horse rabbit serum used in this manner gave a precipitate with normal horse serum up to a dilution of 1:80,000 of the latter.

Results of Immunological Studies

The results of the various bacteriological and immunological studies of the pleural fluids and of the corresponding blood sera are given in Table I. Cultures in rabbit's blood broth and on blood agar plates, as well as mouse inoculations, were negative in the controls and in 18 of the pneumonic fluids obtained from 14 patients. The remaining 13 fluids were infected. Pneumococci corresponding in type to the causative agent of the pneumonia were recovered from 11 fluids, whereas hemolytic streptococci were present as secondary invaders in 2 fluids from Case 15.

From a study of Table I it will be seen that neither antigen nor anti-

[3] Obtained through the courtesy of Dr. Augustus B. Wadsworth, Laboratories of the New York State Department of Health.

[4] Prepared by Dr. T. F. Hunnicut.

Explanation of Table I

Day of puncture and *Day of crisis.* The figures represent the number of days after the onset of the pneumonia.

Days under the heading *Serum treatment* represents all the days during which treatment was given (the figures are inclusive).

Horse serum and *Agglutinins.* The figures represent the weakest dilution of fluid or blood sera in which reactions were observed.

Protection. The titer is represented thus:

+ = protection against 10 or 100 lethal doses.

++ = protection against 1000 or 10,000 lethal doses.

+++ = protection against 100,000 or 1,000,000 lethal doses.

++++ = protection against 10,000,000 or more lethal doses.

Precipitins and *Precipitinogens.* +, + +, etc., represent the intensity of the reaction, from +, indicating a definite but slight precipitate with a cloudy supernatant fluid to + + + +, indicating a clear supernatant fluid and a heavy sediment.

Abbreviations

D.	= died.	Str. h.	= Streptococcus hemolyticus.
P.M.	= post mortem.	Mening.	= meningococcus.
Neg.	= negative.	I	= Pneumococcus Type I.
—	= not done.	II	= Pneumococcus Type II.
Pn.	= pneumococcus.	III	= Pneumococcus Type III.

Case No.	Pneumococcus type	Day of crisis	Day of puncture	Serum treatment		pH	Culture	Mouse inoculation	Pleural fluids Concentration...						
									Horse serum	Protection			Agglutins		
										I	II	III	I	II	I...

§...

I. Patients rec...

				cc.	days										
3	III	10	3	0	—		Neg.	Neg.	—	—	—	—	0	0	0
12	I	5	4	0	—	8.0	Neg.	Neg.	—	—	—	—	0	0	0
2	I	5	5	0	—	7.2	Neg.	Neg.	—	0	—	+	0	0	1
9	I	10	10	0	—	8.0	Neg.	Neg.	—	+	0	—	0	0	0
			18			7.7	Neg.	Neg.	—	++	0	—	8	0	0
5*	III	7	21	0	—	7.6	Neg.	Neg.	—	0	+++	++	0	2	1

II. Patients treated with Felton's...

6	II	4	4	322	2–4	7.8	Neg.	Neg.	400	—	—	—	16	32	0
14	I	3	10	70	2	8.0	Neg.	Neg.	100	++	+	—	0	0	0
11	II	6	9	346	2–5	7.6	Neg.	Neg.	50	++	+	—	0	0	0
4	I	5	33	174	4–5	—	Neg.	Neg.	—	+	++	—	0	0	0
15	II	—	9	264	3–5	8.0	Neg.	Neg.	200	—	++	—	16	32	0
			13			8.0	Neg.	Neg.	100	0	0	—	2	8	0
10	I	18	14	183	13–14	8.2	Neg.	Neg.	400	+++	++	0	0	8	0
			17			8.0	Neg.	Neg.	400	+	++	0	8	32	0

III. Patients tr...

7	I	—	15	53	2	8.0	Neg.	Neg.	50	++	0	0	2	0	0
16	XVIII	7	5	127	2–3	7.7	Neg.	Neg.	200	—	—	—	Mening. 1:8		
			7			8.0	Neg.	Neg.	50	—	—	—	Mening. 1:8		
17	II	3	4	75	2	7.8	Neg.	Neg.	100	—	—	—	0	0	0

I...

I. Patients rec...

18	I	D.4	2	0	—	7.4	Pn. I	Pn. I	—	0	0	0	0	0	0
13	III	D.8	7	0	—	7.8	Pn. III	Pn. III	—	—	—	0	0	0
1	II	D.18	17	0	—	6.4	Pn. II	Pn. II	—	—	—	0	0	0
8	I	—	12	0	—	5.6	Pn. I	Pn. I	—	0	—	—	0	0	0
			15			5.6	Pn. I	Pn. I	—	0	—	—	0	0	0

II. Patients treated with Felto...

18	I	D.4	P.M.	305	3–4	7.2	Pn. I	Pn. I	200	0	0	0	0	8	0
15	II	—	22	264	3–5	7.2	Str. h.	Str. h.	10	0	+	0	0	4	0
			23			5.4	Str. h.	Str. h.	100	0	0	0	0	2	0
19	I	—	10	140	4–5	7.2	Pn. I	Pn. I	400	0	++	—	0	8	0
			13			7.0	Pn. I	Pn. I	400	0	+	—	0	8	0
			14			6.3	Pn. I	Pn. I	200	0	++	—	0	4	0
			19			6.3	Pn. I	Pn. I	200	0	++	0	0	2	0

III. Patients t...

| 7 | I | — | 26 | 53 | 2 | 6.4 | Pn. I | Pn. I | — | — | — | — | — | 0 | |

Nor...

20	Rheumatic pleurisy					7.8	Neg.	Neg.	—	0	0	0	0	0	
21	Cardiac decompensation					7.8	—	—	—	—	—	—	—	0	0
22	Pleurisy with effusion					8.2	Neg.	Neg.	—	—	—	0	0	0	
						7.8	Neg.	Neg.	—	—	—	0	0	0	

* This patient received skin tests with the specific polysaccharides of Types I, II, and III pneum...

				Blood culture	Blood serum (Concentration of antigen and antibodies)									
	Precipitinogens				Horse serum	Protection			Agglutinins			Precipitins		
III	I	II	III			I	II	III	I	II	III	I	II	III
...m treatment														
0	0	0	0	Neg.	—	0	0	0	0	0	0	0	0	0
0	0	0	0	Neg.	—	+	0	+	8	8	0	0	0	0
+	−	−	−	Neg.	—	++	0	0	8	0	0	++	0	0
0	0	0	0	Neg.	—	++	0	0	8	0	0	++	0	0
0	0	0	0	Neg.	—	++	+++	+++	0	64	64	0	+++	++
+	0	0	0	Neg.	—	+							+++	++
...ed antibodies (Types I and II)														
++	0	0	0	Neg.	1600	+++	++	—	16	32	4	+++	+++	0
0	0	0	0	Neg.	—	—	—	—	—	—	—	—	—	0
0	0	0	−	Neg.	—	+	++	—	0	0	0	+	+	0
++	0	0	0	Neg.	1600	0	0	—	4	8	0	++	+++	0
+	0	0	0	Neg.	1600	0	+	—	32	64	0	++	+++	0
+	0	0	0	Pn. I	1600	++++	++	—	32	64	0	++	+++	0
+	0	0	0	Neg.	1600	+++	++	—	16	32	0			
non-specific sera														
0	0	0	0	Neg.	—	+	—	—	4	0	0	—	—	—
−	−	−	−	Neg.	800	—	—	—	Mening. 1:1600			—	—	—
−	−	−	−	Neg.	800	—	—	—	Mening. 1:1600			—	—	—
0	0	0	0	Neg.										
...ls														
...rum treatment														
0	0	++++	0	0	Pn. I / Pn. III	—	0	0	0	0	0	0	0	0
0	0	0	0	++++	Pn. III									
0	0	0	++++	0										
0	0	++++	0	0										
0	0	++++	0	0										
...ated antibodies (Types I and II)														
0	0	++++	0	0	Pn. I	400+	+++	+++	+	32	64	4	0	++++	0
+	0	0	0	0	Neg.	1600	0	—	—	2	8	0	+	0	0
0	0	0	0	0	Neg.	800	—	+	—	2	4	0	0	0	0
0	0	++++	0	0	Neg.	400	++++	++	—	8	4	0	0	0	0
0	0	++++	0	0	Neg.	400	+++	+	—	2	4				
0	0	++++	0	0	Neg.									
0	0	++++	0	0	Neg.									
non-specific sera														
0	0	++++	0	0	Neg.									
...Fluids														
0	0	0	0	0	Neg.	—	0	0	0	0	0	0	0	0
0	0	0	0	0		—	—	—	0	0	0	0	0	0
0	0	0	0	0	Neg.									
0	0	0	0	0	—									

bodies could be demonstrated by precipitin tests in the sterile pneumonic fluids during the acute stage of the disease when no specific sera had previously been given. At this time antibodies could not be found in the blood of these patients. At the time of crisis or later antibodies corresponding to those found in the blood sera were demonstrated in the sterile fluids of the untreated cases. Sterile fluids obtained from patients who had received therapeutic sera were shown to contain horse serum and antibodies corresponding to those of the therapeutic sera. Similar antibodies were found in the blood of these patients.

Fluids infected with pneumococci always showed homologous soluble specific substance, as demonstrated by the specific precipitate with the homologous antipneumococcic serum. In the cases that were given antisera containing the homologous and heterologous antibodies, horse serum and heterologous antibodies could be demonstrated along with homologous antigen. The blood, however, contained both homologous and heterologous antibodies. When infection was due to a secondary invader (Case 15), homologous pneumococcic antibodies could be demonstrated in the pleural fluid. Occasionally no antibodies could be demonstrated in fluid when the serum showed a good titer. In one instance (Case 7), when a fluid, sterile early in convalescence, later became infected, actively acquired antibodies for the homologous type were demonstrated on the former occasion and antigen, but no antibodies, on the latter. The tests on the control fluids in the nonpneumonic patients were entirely negative.

The quantitative relationships between the antibodies in the fluids and those in the corresponding sera were not always constant. The protective titer is difficult to compare, but in half of the instances in which protection for the same organisms was demonstrated in both the serum and fluid the titer was found to be the same. In the remaining instances, with one exception in which more protection was found in the fluid, the serum protected in only 1 or 2 dilutions higher (against 10 to 100 times the number of lethal doses of pneumococci) than the corresponding fluid.

The ratio of the agglutinin titers in the fluids to those in the sera varied from 2:1 to 1:32. In 19 instances where corresponding agglutinins were demonstrated in both fluid and serum, the ratio of the titer

in the fluid to that in the sera was 1:1 or 1:2 in 10 instances; in 4 others, it was 1:4 or 1:8; in 3, it was 1:32; and, in the remaining 2, it was 2:1.

The titer of horse serum, using the same anti-horse rabbit serum throughout, showed similar relations between the fluid and blood. Of 12 instances where a comparison was possible the ratio of the titer in the fluid to that in the serum was 1:1 in 2 instances; 1:4 in 5; 1:8 or 1:16 in 4; and 1:160 in a single instance.

DISCUSSION

Numerous studies have been made on absorption of fluids, fats, particulate matter, and bacteria from the pleural cavity (28). Little mention, however, has been made of the passage of materials from the circulation into the pleural cavity. Furthermore, as far as could be ascertained, the only direct reference to the presence of specific antibodies in pneumonic pleural fluids are those referred to above (9, 10, 13). It has here been demonstrated that antibodies actively produced by the patient or passively introduced into the blood stream appear in the pleuritic effusion of patients with lobar pneumonia, whether such effusions are sterile or infected. As a rule, the concentration of horse serum and of antibodies in the fluids has been either the same or, more commonly, lower than in blood serum. This is not in accord with the findings of Couraux (29) and Paraskevopoulos (30), who each found more antibodies against the tubercle bacillus in pleural fluid than in the blood, but agrees with the finding in Pepper's case (15) where the ratio of the titer of typhoid agglutinins in the fluid to that in the serum was 4:5. Pinner and Moerke (17) found that the blood averaged 27 per cent more globulin than the tuberculous pleural effusions which they studied, although there were individual instances where the fluid contained more globulin than the serum. These findings are significant since the intimate association of antibodies with serum globulin is well recognized.

These observations cannot, of course, be interpreted as indicating that the normal pleura is permeable to immune bodies. That injury to the pleura through the inflammatory processes in the lung renders it more permeable is very likely in view of the findings of Flexner and his coworkers (21) in relation to the passage of immune substances into the cerebrospinal fluid.

It appears that the presence of antibodies in the effusion does not necessarily prevent its subsequent infection by the homologous organisms. When such infection does occur, soluble specific substance appears in the fluid and the homologous antibody can no longer be demonstrated; but such horse serum and heterologous antibodies as may have been introduced can still be found. This agrees with the findings of Cole (11) already mentioned.

SUMMARY AND CONCLUSIONS

Pleuritic exudates from patients with lobar pneumonia may be sterile or infected. Sterile fluids, at or about the time of crisis, contain actively acquired antibodies similar to those in the blood serum. Infected fluids do not contain such antibodies, presumably because of the presence in them of large amounts of soluble specific substance. Sterile fluids from patients treated with immune sera have both horse serum and antibodies similar to those injected. Infected fluids from serum-treated cases contain horse serum and such heterologous antibodies as were contained in the therapeutic sera together with homologous soluble specific substance. The concentration of horse serum and antibodies in pneumonic fluids is usually the same or somewhat less than that of the corresponding blood sera.

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TOXIN-ANTITOXIN REACTION WITHOUT NEUTRALIZATION

By JULES FREUND, M.D.

(From The Henry Phipps Institute, University of Pennsylvania, Philadelphia)

It seems probable that the recent progress in the study of antigen and antibody reactions, particularly of agglutination and phagocytosis, is due to the circumstance that they can be studied on the surface of particulate matter (1). It seems similarly desirable to investigate the reactions between toxins and antitoxins on the surface of simple particles. The experiments to be reported are a study of how toxin and antitoxin react when they are in contact, the one adsorbed on collodion particles and the other in solution.

EXPERIMENTAL

Collodion particles were prepared according to Loeb's (2) method as modified by Kunitz (3). Collodion U.S.P. is poured in 2 l. of water while the mixture is being stirred. The collodion that is precipitated is washed with water several times, then pressed by hand and then between filter papers. The dry collodion is cut into small pieces and dissolved in a small amount of acetone at about 40°C. in a beaker. The beaker containing the collodion solution is placed in water at a temperature of about 40°C. Then while the acetone-collodion solution is stirred mechanically a mixture of water and acetone (three parts of water and one part of acetone) is added to it from a funnel through a capillary pipette, drop by drop, until a heavy gelatinous mass is formed. The supernatant cloudy fluid is poured into a flask and diluted with water. When the water is added to the collodion suspension more collodion particles are formed. The acetone can be removed from the suspension either by vacuum distillation at from 35° to 40°C. or by washing the particles with the aid of the centrifuge. The gelatinous mass of collodion from which the supernatant suspension was poured is dissolved in acetone and mixed with water-acetone mixture and treated as described above. The large particles are separated by centrifugalization and discarded. The final suspension contains particles of about the size of staphylococci.

Experiments with Diphtheria Toxin[1]

In previous papers (4) it was reported that collodion particles treated with diphtheria toxin are flocculated by diphtheria antitoxin and that

[1] The toxin and antitoxin preparations were obtained through the courtesy of Professor Karl F. Meyer and the H. K. Mulford Co.

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when collodion particles treated with diphtheria toxin are injected into the skin of guinea pigs inflammation follows, which can be prevented by the simultaneous injection of diphtheria antitoxin.

In the present study the question was investigated whether toxin is neutralized when (1) collodion particles treated with toxin are suspended in antitoxin, and (2) when the order of treatment of particles is reversed, *i.e.*, when they are treated first with antitoxin and then with toxin.

The M.L.D. of the toxin was 0.003 cc. The unconcentrated antitoxic horse serum contained 500 units per cubic centimeter.

1. Treatment of Particles with Diphtheria Toxin.—1 cc. of a heavy suspension of collodion particles in saline solution was added drop by drop to 5 cc. of 1:10 dilution of diphtheria toxin. The mixture was gently shaken for 5 minutes and centrifugalized. The supernatant fluid was discarded, the wall of the tube wiped with filter paper, the particles suspended in 40 cc. of saline and washed three times. (The last washing fluid gave negative skin test in guinea pigs.) The particles were suspended in 0.5 cc. saline, and 0.25 cc. injected into the skin of a guinea pig or rabbit.

2. Treatment of Particles with Diphtheria Toxin and Antitoxin.—After the particles had been treated as described above, they were suspended in 5 cc. of a 1:10 dilution of diphtheria antitoxin, gently shaken for 5 minutes, washed three times, suspended in 0.5 cc. of saline; 0.25 cc. of the suspension was used for skin test.

3. Treatment of Particles with Diphtheria Toxin and Tetanus Antitoxin.—The same amount of the collodion suspension and the same amount of the diluted diphtheria toxin was used as in Experiment 1. After the particles were washed three times they were suspended in 5 cc. of a 1:10 dilution of tetanus antitoxin, shaken 5 minutes, centrifugalized and washed three times. The particles were then suspended in 0.5 cc. saline and 0.25 cc. injected into the skin of a rabbit or guinea pig.

4. Treatment of Particles with Diphtheria Antitoxin and Toxin.—Treatment of particles as described under 2 but in reversed order, first with antitoxin, then with toxin.

5. Treatment of Particles with Tetanus Antitoxin and Diphtheria Toxin.—The particles were treated as described under 3, but in reversed order.

Untreated collodion particles cause a nodule when injected into the skin of guinea pigs or rabbits; the skin over the nodule is not discolored. In the presence of the adsorbed toxin the nodule is usually larger, the skin becomes red or purple and it may undergo superficial necrosis. The discoloration of the skin usually appears 2 days after inoculation and it may increase for 2 days. Positive skin reactions

were observed only in guinea pigs weighing more than 500 gm. and not all guinea pigs used for testing reacted. Rabbits reacted more

Rabbit

1. Diphtheria antitoxin + toxin

2. Diphtheria toxin alone

Redness: 21 x 16 mm.
Necrosis: 10 x 7 mm.

Redness: 9 x 8 mm.
Necrosis: 3 x 2.5 mm.

Guinea Pig

1. Diphtheria antitoxin + toxin

2. Diphtheria toxin alone

Redness: 18 x 15 mm.
Necrosis: 7 x 6 mm.

Redness: 8 x 6 mm.
Necrosis: 4 x 3 mm.

DIAGRAM 1. Area of redness and necrosis at site of injection of treated collodion particles.

uniformly. It was found that collodion particles treated with toxin and then with antitoxin produced a nodule over which the skin remained normal. With toxin and normal serum the reaction was either the same as with toxin alone or weaker. Particles treated first with

normal serum or tetanus antitoxin and then with toxin caused no reaction. When, however, collodion particles treated first with diphtheria antitoxin and then with toxin were injected the skin over the nodule became inflamed and necrotic; the inflammation was strikingly more intense than with particles treated with toxin alone.

Diagram 1 illustrates the areas of redness and necrosis in rabbits and guinea pigs caused by injections of treated collodion particles. These experiments may be summarized as follows:

	On the collodion particles		*In solution*		*Collodion particles*
I	Diphtheria toxin	+	Diphtheria antitoxin	:	Not toxic
II	Diphtheria toxin	+	Tetanus antitoxin	:	Toxic
III	Diphtheria antitoxin	+	Diphtheria toxin	:	Toxic
IV	Tetanus antitoxin	+	Diphtheria toxin	:	Not toxic

Experiment with Tetanus Toxin

The M.L.D. of the toxin was 0.0001 cc. The unconcentrated antitoxic horse serum contained 700 units per cubic centimeter.

In the first experiment 1 cc. of the suspension of collodion particles was treated with 5 cc. of a 1:10 dilution of tetanus toxin. After the particles had been washed and suspended in 0.5 cc. of saline 0.1 cc. of the suspension was injected into the right hind leg of a mouse. The mouse had local tetanus in 2 days and died in 4 days after the injection. Mice injected with the last washing fluid remained free from tetanus. When 0.1 cc. of the suspension of collodion particles treated with toxin was suspended in 5 cc. of tetanus antitoxin diluted from 1:10 to 1:50,000 and washed no symptoms were produced in mice. Diphtheria antitoxin had no effect on the toxicity of the particles.

After it had been found that tetanus toxin is adsorbed on collodion particles and is neutralized by tetanus antitoxin, but not affected by normal horse serum or diphtheria antitoxin, experiments were performed to ascertain whether the order of treatment would influence the result, *i.e.*, whether antitoxin *adsorbed* on the particles would neutralize the toxin. Therefore, particles were treated: (*a*) first with tetanus antitoxin and then with tetanus toxin; (*b*) first with diphtheria antitoxin and then with tetanus toxin.

In these experiments the dilutions of serums and toxin were varied. With antitoxin dilution 1:1 and toxin dilution 1:1 or 1:10, with antitoxin dilution 1:10 and toxin dilution 1:1 or 1:10 the mice in the experiments with diphtheria antitoxin and tetanus toxin showed no symptoms at all; in the experiments with tetanus antitoxin and tetanus toxin they either died or had severe symptoms of local

tetanus. When antitoxin was used in dilution 1:10 and toxin 1:100 the mice in both types of experiment remained free from tetanus.

When these results are summarized they are seen to parallel the results of the similar experiment with diphtheria toxin.

On the collodion particles		In solution	Collodion particles
I	Tetanus toxin	+ Tetanus antitoxin	: Not toxic
II	Tetanus toxin	+ Diphtheria antitoxin	: Toxic
III	Tetanus antitoxin	+ Tetanus toxin	: Toxic
IV	Diphtheria antitoxin	+ Tetanus toxin	: Not toxic

When the tetanus toxin was fresh (1 month old) the collodion particles treated with the antitoxin and then the toxin were either as toxic or even more toxic than particles treated with toxin alone. When the toxin was 8 months old the particles treated with antitoxin were not so toxic as particles treated with toxin alone. In several experiments the particles were treated with normal horse or rabbit serum, and in one experiment with egg white diluted 1:10 before they were mixed with tetanus toxin. Such particles remained non-toxic like those treated first with diphtheria antitoxin and then with tetanus toxin.

The experiments with tetanus toxin showed that:

1. Collodion particles adsorbed tetanus toxin and retained it in salt solution, but the adsorbed toxin was at least in part released in the animal.

2. The adsorbed toxin was neutralized by suspending the particles coated with toxin in a dilution of antitoxin. It is not clear from this experiment whether the neutralization occurs on the collodion particles before injection, for it is possible that the toxin is neutralized after both toxin and antitoxin have been released in the animal.

3. The neutralization is specific.

4. If collodion particles are treated first with diphtheria antitoxin or normal horse serum or egg white and then with tetanus toxin, the particles are not toxic for mice.

5. If collodion particles are treated first with tetanus antitoxin then with tetanus toxin the particles become toxic for the animal. Since particles first treated with diphtheria antitoxin and then with tetanus toxin are not toxic, the toxicity must be due to the action of tetanus antitoxin upon the tetanus toxin.

These results can be explained by assuming that the antitoxin adsorbed on the particles is able to adsorb toxin without being able to neutralize it. Since the toxin adsorbed on particles that had been previously treated with antitoxin remains unneutralized, the question arises whether or not the adsorbed toxin is susceptible to neutralization by antitoxin. It was found that when particles treated first with antitoxin and then with toxin were treated again with antitoxin they produced no symptoms in mice.

The minimal lethal dose of tetanus toxin that had been in contact with particles treated either with tetanus or diphtheria antitoxin was found to be the same and hardly different from that of the toxin before adsorption. Apparently the particles adsorb so little toxin that the difference might escape detection.

Experiments with Botulinus Toxin

These experiments did not produce consistent results. The toxin was adsorbed and retained on collodion particles; however in some experiments the collodion particles treated with toxin and antitoxin proved to be toxic. When collodion particles were treated first with tetanus or *botulinus* antitoxin and then with toxin, and injected into mice, the results varied so much that no conclusions could be drawn.

DISCUSSION

Toxins are adsorbed by various colloids: charcoal, colloidal iron hydroxide, kaolin (5). In the present study a negatively charged colloid, a suspension of collodion particles, was used for the adsorption of toxins and antitoxins. Collodion particles seem to have the advantage over other adsorbents that collodion is insoluble in water, is changed in the animal only with difficulty, if at all, is suitable for studying flocculation reactions and can be used in cataphoretic studies. Loeb, Northrop, Hitchcock and Kunitz (6) have used collodion as adsorbent in studying properties of protein that cannot be observed in solution. In connection with a study on the surface properties of tubercle bacilli, I found that collodion particles coated with egg white are flocculated by anti-egg white serum (7). Jones reported that collodion particles can be coated with various proteins and flocculated with precipitin serums (8). Mudd, Lucké, McCutcheon and Strumia have studied the mechanism and have also demonstrated

that collodion particles treated with proteins and specific antiserum are phagocytosed like bacteria treated with bacteriotropins (9). I have reported (4) that collodion particles treated with tetanus toxin are *not* flocculated by tetanus antitoxin, whereas collodion particles coated with diphtheria toxin are flocculated by the homologous antitoxin. Bedson (10) found that herpes virus can be adsorbed and neutralized on the surface of collodion particles.

In the present study it was found that tetanus, diphtheria and *botulinus* toxins and antitoxins can be adsorbed upon collodion particles, and that the adsorbed tetanus and diphtheria toxins can be neutralized by their corresponding antitoxins. When the order of treatment was reversed an interesting phenomenon was observed: although treatment with a heterologous toxin of collodion particles coated first with antitoxin resulted in non-toxic particles, treatment with the hemologous toxin resulted in toxic particles. The results of the experiments showing this cross-specificity may be summarized as follows:

	On collodion particles	In solution	Collodion particles
A	Tetanus antitoxin	+ Tetanus toxin	: Toxic
B	Diphtheria antitoxin	+ Tetanus toxin	: Not toxic
A'	Diphtheria antitoxin	+ Diphtheria toxin	: Toxic
B'	Tetanus antitoxin	+ Diphtheria toxin	: Not toxic

It is very probable that the particles treated with non-specific serum are not able to adsorb toxin because the protein on the particles does not adsorb toxins, whereas in the specific combination the antitoxin antibody is able to adsorb more toxin than it can neutralize. This observation can be compared with that of Jones, who found that "collodion particles exposed to immune serum and subsequently washed fail to agglutinate in the presence of antigen."

There is a certain—perhaps superficial—similarity between this apparently paradoxical phenomenon and the Bordet-Danysz reaction in regard to the importance of the order of mixing the reagents. Danysz found that when to a definite amount of antitoxin a definite quantity of toxin is added the mixture is more toxic if the latter (diphtheria toxin or ricin) is added fractionally than when the whole amount is added at once. However, if the antitoxin is added fractionally to the

These results can be explained by assuming that the antitoxin adsorbed on the particles is able to adsorb toxin without being able to neutralize it. Since the toxin adsorbed on particles that had been previously treated with antitoxin remains unneutralized, the question arises whether or not the adsorbed toxin is susceptible to neutralization by antitoxin. It was found that when particles treated first with antitoxin and then with toxin were treated again with antitoxin they produced no symptoms in mice.

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When collodion particles are treated first with toxin then with antitoxin the antitoxin is in excess and with the reversed order of treatment the toxin is in excess.

3. Another possibility is that neutralization occurs only if the antitoxin is in the surface layer of the collodion-toxin-antitoxin complex so that the surface of the complex has the properties of the antitoxin. At present it is thought that in agglutination of bacteria or red blood cells by immune serum the surface of the cell is coated entirely or partially with the antibody and the complex has the properties of the antibody (denatured globulin, insoluble in salt solution) (11). The suggestion that neutralization depends upon establishing the surface properties of the antitoxin for the toxin-antitoxin complex is also supported by the flocculation reaction, which occurs in neutral or almost neutral mixture of diphtheria and tetanus toxins and antitoxins.

The second and third possibilities are not mutually exclusive but may both operate at the same time. Thus on particles treated first with antitoxin and then with toxin the excess of toxin present may be more effective because it is situated largely at the outer surface of the collodion-antitoxin-toxin aggregate and is then able to come into contact with the tissue cells for which it is toxic. Similarly on particles treated first with toxin and then with antitoxin the excess of antitoxin may act in part by coating the outer surface of the collodion-toxin aggregate and thus preventing contact between toxin and tissue cells. It is also possible that neutralization of toxin by antitoxin is conditioned by specific adsorption. The process of the essential neutralization and the specific adsorption of toxin and antitoxin may follow different rules; the former may occur according to definite, and the latter, according to variable proportions.

The observations reported here showing the significance of the order of treatment of collodion particles in neutralization, together with the similar observations made by Jones with precipitinogen and precipitin establish an analogy between the toxin-antitoxin reaction and other antigen-antibody reactions, particularly agglutination and precipitation. This similarity is strengthened by experiments showing that tannin adsorbed on red blood cells mediates agglutination, promotes phagocytosis, prepares red blood cells for lysis by complement

toxin the resulting mixture is not more toxic than when the entire amount of antitoxin is added at once. In the reaction on collodion particles as well the anomalous excess of toxicity results only if toxin is added to antitoxin but not with the reversed order.

The question naturally arises why the particles treated with antitoxin and then with toxin are toxic, when the opposite order of treatment yields non-toxic particles. In the experiments with tetanus toxin the particles treated with antitoxin and toxin are not more toxic than those treated with toxin alone. Therefore it might be possible that while the particles treated with antitoxin are in contact with toxin they lose their previously adsorbed antitoxin film and adsorb toxin. However, when particles treated with antitoxin and toxin are heated at 55°C. for $\frac{1}{2}$ hour (to destroy the toxin) antitoxin is released when injected into mice, showing that the antitoxin was retained during the contact with toxin. 1 cc. of a suspension of such particles protected mice against one lethal dose of toxin. In the diphtheria toxin experiments particles treated with antitoxin and toxin are even more toxic than those treated with toxin alone. Apparently a specific binding between the toxins and antitoxins is not sufficient for neutralization. Three possibilities may be considered:

1. It is possible that in the toxin and antitoxin molecules there are two groups participating in the neutralization reaction as was assumed by Ehrlich. The combination of collodion, antitoxin and toxin may be toxic because when the antitoxin is adsorbed on collodion it has only one free group, which combines with the toxin, the haptophor group, but the neutralizing ergophor group is fixed to the particle and not free to act upon, to neutralize, the toxin.

2. The second, perhaps the more likely, explanation is the following: It is well established that a definite number of bacteria or red blood cells can adsorb various amounts of agglutinins from a solution containing agglutinins depending upon the concentration (titer) of the solution, temperature, etc., and that they can adsorb a considerably greater number of units of agglutinins than is necessary for agglutination (Bordet). It is possible that toxins adsorbed on collodion particles adsorb more units of antitoxins that is necessary for neutralization. Similarly it is possible that antitoxins adsorbed on collodion particles adsorb more units of toxins than they are able to neutralize.

When collodion particles are treated first with toxin then with antitoxin the antitoxin is in excess and with the reversed order of treatment the toxin is in excess.

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and that it combines with and detoxifies toxins adsorbed on collodion particles (12).

CONCLUSIONS

1. Collodion particles adsorb diphtheria or tetanus or *botulinus* toxins. These toxins are retained on the particles when washed but are at least in part released in the animal.

2. The adsorbed toxins are neutralized by adsorption of the corresponding antitoxins but are unaffected by other serums.

3. When collodion particles are treated first with tetanus antitoxin, then with diphtheria toxin, they are not toxic, but they become toxic when they are treated first with diphtheria antitoxin, then with the diphtheria toxin. Similarly when collodion particles are treated first with diphtheria antitoxin and then with tetanus toxin, they do not become toxic, but they become toxic when they are treated with tetanus antitoxin and tetanus toxin.

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A NOTE ON THE SPECIFIC AGGLUTINATION OF PNEU-MOCOCCUS TYPES I, II AND III

By JOHN F. ENDERS, Ph.D.

(From the Department of Bacteriology and Immunology, Harvard University Medical School, Boston)

In a preceding paper we presented evidence for the existence of a type-specific substance (i.e. the so called A substance) in Pneumococcus Type I distinct from the specific carbohydrate of Avery and Heidelberger. During the course of the experiments described in that communication it was noted that untreated Antipneumococcus Type I rabbit sera which contained no precipitin against the purified carbohydrate, nevertheless exhibited agglutinin titre against the homologous organism at least as high as 1/240. This observation suggested the possibility that the S organism might contain an agglutinogen unrelated to the specific carbohydrate. Moreover, since the same antisera were found to contain precipitin reacting with the type-specific A substance, the hypothesis that the observed agglutination might also be caused by the A antibody seemed not unreasonable. The experiments to be described not only lend further support to this hypothesis, but confirm the original observation concerning the agglutination of Pneumococcus Type I by antisera which fail to react by visible precipitation with the specific carbohydrate as well as extend these findings to the smooth organisms of Types II and III.

Technique

Preparation of the Antigens.—Cultures of pneumococci Types I, II and III which had been recently passed through mice were grown in phosphate buffer hormone broth. The growth after incubation at 37°C. for 18 hours was removed by centrifuging and resuspended in a volume of physiological salt solution equivalent to that of the decanted broth. The saline suspension was then divided into three portions. To the first, sufficient formalin was added to give a concentration of 0.2 per cent. The formalinized suspension was then maintained at 37°C. for 14 to 16 hours.

The reaction of the second portion of the saline suspension was adjusted to about pH 5 by means of acetic acid and subsequently maintained at 100°C. in the Arnold for 40 minutes. The acidified suspension was allowed to stand at 37°C. for 14 to 16 hours. The pH was then brought to 7 with NaOH.

The third portion was treated with NaOH until a pH of about 8.8 was obtained. It was thereupon subjected to the same physical conditions as the acidified suspension, with final adjustment of the pH to 7.

In most of the experiments these suspensions were employed within a day or two of preparation. In a few cases older antigen solutions were used which had been kept in the ice box for from 1 to 2 weeks.

Examination of smears of the heated suspensions stained by Gram's method revealed no gross changes in the typical morphology of the organism, nor was its ability to retain the gentian violet impaired.

Preparation of Antisera.—The antipneumococcus horse sera were typing sera secured in the cases of Type I and Type II from the Massachusetts State Antitoxin Laboratory and in the case of Type III from the New York State Laboratory at Albany.

The type-specific antipneumococcus rabbit sera were produced by injection of rabbits with formalinized suspensions of pneumococci. These untreated antisera will hereafter be referred to as "unabsorbed."

The antisera which in this paper are designated as "absorbed" were obtained by repeated precipitation of the type-specific unabsorbed antisera described above with the homologous specific carbohydrate purified according to the method of Heidelberger and his associates. The details of the technique employed in the removal of the S antibody are described in a previous communication (1).

A comparison of the precipitating power of the horse antisera used in the following experiments before and after absorption with the carbohydrate is shown in Table I. The absorbed rabbit sera used in the experiments with Pneumococcus Type I and Type II exhibited the same inability to precipitate in the presence of the specific carbohydrate.

EXPERIMENTAL

The experiments which indicated the presence of a type-specific agglutinogen in Pneumococcus Types I, II and III distinct from the specific carbohydrate consisted simply in testing the three antigens prepared in the manner already described against the homologous unabsorbed or untreated antiserum and the same antiserum from which the antibody reacting with the specific carbohydrate had been largely removed by repeated precipitation at the optimum point of flocculation with the

TABLE I

Effect of Absorbing Pneumococcus Antisera with the Specific Carbohydrate

Antiserum	Antigen	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000	1/2,000,000	1/4,000,000	1/8,000,000
Antipneumococcus I horse serum unabsorbed	Specific carbohydrate Pneumococcus I	++++	++++	++++	+++++	++	+	+	
Antipneumococcus I horse serum absorbed	Specific carbohydrate Pneumococcus I	±?	–	–	–	Not done	Not done	Not done	
Antipneumococcus II horse serum unabsorbed	Specific carbohydrate Pneumococcus II	++++	++++	++++	+++++	+	+	–	
Antipneumococcus II horse serum absorbed	Specific carbohydrate Pneumococcus II	±?	–	–	–	–	–	–	
Antipneumococcus III horse serum unabsorbed	Specific carbohydrate Pneumococcus III	++++	++++	++++	+++++	++	++	+	
Antipneumococcus III horse serum absorbed	Specific carbohydrate Pneumococcus III	±	–	–	–	–	–	–	

The results recorded above were those obtained from ring tests read after standing 2 hours at room temperature.

TABLE II

Agglutination of Pneumococcus I in Unabsorbed and Absorbed Antisera

Antiserum	Antigen	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/312	1/1,024
Antipneumococcus I horse serum unabsorbed	Formalinized Pneumococcus I	++++	++++	++++	++++	++++	++++	+++	++±	—	—
Antipneumococcus I horse serum absorbed	Formalinized Pneumococcus I	++++	++++	++++	++++	++++	++++	++	+	—	—
Antipneumococcus I horse serum unabsorbed	Pneumococcus I boiled pH 5	++++	++++	++++	++++	++++	++±	++	+	—	—
Antipneumococcus I horse serum absorbed	Pneumococcus I boiled pH 5	+++±	++++	++++	++++	++++	++±	+	—	—	—
Antipneumococcus I horse serum unabsorbed	Pneumococcus I boiled pH 8.8	++++	++++	++++	++++	++++	+++	+	+	—	—
Antipneumococcus I horse serum absorbed	Pneumococcus I boiled pH 8.8	++++	++++	++++	++++	++++	+++	++±	+	++	—
Normal horse serum	Formalinized Pneumococcus I	±	+	—	—	—	—	—	—	—	—
Normal horse serum	Pneumococcus I boiled pH 5	±	±	+	±?	—	—	—	—	—	—
Normal horse serum	Pneumococcus I boiled pH 8.8	±	—	—	—	—	—	—	—	—	—

Tubes in agglutination tests contained equal parts of diluted antiserum and antigen suspension. Readings after 16 hours in water bath at 53°C.

purified polysaccharide. To show that the agglutination obtained with the absorbed antisera was type-specific, cross-agglutination tests with the heterologous absorbed antisera were performed. No agglutination of the heterologous organism was observed in the presence of these sera.

In Table II are recorded the results of an experiment in which the agglutinin titre of Type I pneumococcus unabsorbed and absorbed antisera were determined in the presence of suspensions of Pneumo-

TABLE III

Effect of Heating the Specific Carbohydrates of Pneumococcus I, II and III at pH 8.8

Dilution of type-specific anti-serum.... Antigen	1/10,000	1/100,000	1/1,000,000	1/2,000,000	1/4,000,000	1/8,000,000
Pneumococcus I specific carbohydrate unboiled	++++	++++	++	+	±	±?
Pneumococcus I specific carbohydrate boiled at pH 8.8	++++	++++	+±	+	±	—
Pneumococcus II specific carbohydrate unboiled	++++	+++±	+	+	—	—
Pneumococcus II specific carbohydrate boiled at pH 8.8	++++	+++±	+±	±	—	—
Pneumococcus III specific carbohydrate unboiled	++++	++++	++	+±	+	±
Pneumococcus III specific carbohydrate boiled at pH 8.8	++++	++++	++±	++	+	±

Ring test readings after 2 hours at room temperature.

coccus Type I after formalinization and after boiling at acid and alkaline reactions. The experiment was repeated using an Anti-pneumococcus I rabbit serum. Results of identical character were obtained.

A comparison of the titres of the unabsorbed and absorbed sera show that (1) the removal of a large proportion of the specific carbohydrate-precipitating antibody does not reduce the agglutinating titre of the serum for Pneumococcus Type I when treated with formalin or boiled at pH 5, and (2) that when this organism is boiled at pH

TABLE IV

Agglutination of Pneumococcus II in Unabsorbed and Absorbed Antisera

Antiserum	Antigen	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1,280	1/2,560
Antipneumococcus II horse serum unabsorbed	Formalinized Pneumococcus II	++++	++++	++++	++++	++++±	+	−	−	−
Antipneumococcus II horse serum absorbed	Formalinized Pneumococcus II	++	+++±	++±	+	+	+±	−	−	−
Antipneumococcus II horse serum unabsorbed	Pneumococcus II boiled pH 5	++++	++++	++++	++++	++++±	++	+±	−	−
Antipneumococcus II horse serum absorbed	Pneumococcus II boiled pH 5	+++	++++	+++	+±	+	+±	−	−	−
Antipneumococcus II horse serum unabsorbed	Pneumococcus II boiled pH 8.8	++++	++++	++++	++++	+++	+±?	−		−
Antipneumococcus II horse serum absorbed	Pneumococcus II boiled pH 8.8	++++	+++	+	+±?	+	−	−		
Antipneumococcus II rabbit serum unabsorbed	Formalinized Pneumococcus II	+++	++++	+++	++	++±	+	+	++	−
Antipneumococcus II rabbit serum absorbed	Formalinized Pneumococcus II	+++	+++	+++±	++	++	++±	+	++	−

Antipneumococcus II rabbit serum unabsorbed	Pneumococcus II boiled pH 5	++++	++++	+++	++	++±	+	±	—	—
Antipneumococcus II rabbit serum absorbed	Pneumococcus II boiled pH 5	+++±	++	+++	++	++	+	±	—	—
Antipneumococcus II rabbit serum unabsorbed	Pneumococcus II boiled pH 8.8	++++	++++	+++±	++	++	—	—	—	—
Antipneumococcus II rabbit serum absorbed	Pneumococcus II boiled pH 8.8	±	—	—	—	±	—	—	—	—
Antipneumococcus II rabbit serum unabsorbed	Pneumococcus II specific carbohydrate	+++	++	+	±					

Tubes in agglutination tests contained equal parts of diluted antiserum and antigen suspension. Readings after 16 hours in water bath at 50°C.

Controls consisting of dilutions of normal horse and rabbit sera were negative.

8.8, it fails to be agglutinated by the absorbed serum. That boiling at this reaction does not entirely destroy its capacity to act as an agglutinable particle in the presence of antiserum containing the S-precipitating antibody is also made clear.

Since, as is shown in Table III, the purified specific carbohydrates of the three pneumococcus types after boiling for 40 minutes at a reaction of pH 8.8 show no definite reduction of their function as precipitating agents, it is reasonable to infer that the constituent in Pneumococcus Type I responsible for the agglutination of the formalinized or acid-boiled organism in the absorbed antiserum and which is rendered inactive by boiling at pH 8.8 is a type-specific agglutinogen differing essentially from the specific carbohydrate.

Employing the same technique, the experiment was again performed using as antigens suspensions of Pneumococcus Type II. The results given in Table IV parallel those obtained for Pneumococcus Type I. Pneumococcus Type II also apparently contains an agglutinogen which is heat-labile at an alkaline reaction.

It will be noted that in the case of Pneumoccoccus Type I the titre of the unabsorbed antiserum was the same for all three suspensions. In the Type II unabsorbed rabbit antiserum the end-point of the titration in which the alkaline-boiled suspension is used falls short of that recorded for the formalinized and acid-boiled antigen. This fact affords further evidence of the presence of an agglutinogen distinct from the specific carbohydrate, since it is probable that the end-point of the alkaline-boiled suspension represents the final effect of the antibody reacting with the specific carbohydrate; while the end-points of the formalized and acid-boiled suspension indicate the quantity at which a second type-specific agglutinin ceases to react. A certain amount of experimental support is given to this hypothesis by the precipitating titre obtained by diluting the unabsorbed anti-Type II pneumococcus rabbit serum and using a constant quantity of the purified specific carbohydrate as antigen. The end-point of such a titration, to be found in Table IV, closely approximates that attained when the alkaline-boiled suspension is added to the same dilutions of the antiserum.

In parallel experiments with Pneumococcus Type III the outcome was somewhat different. The data assembled in Table V show that

TABLE V

Agglutination of Pneumococcus III in Unabsorbed and Absorbed Sera

Antiserum	Antigen	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1,280	1/2,560	1/5,120
Antipneumococcus III horse serum unabsorbed	Pneumococcus III formalinized	++++	+++++	++++	++++	++++	+++++	+++	++	±	—
Antipneumococcus III horse serum absorbed	Pneumococcus III formalinized	++++	++++	++++	+++++	+++++	++	+	—	—	—
Antipneumococcus III horse serum unabsorbed	Pneumococcus III boiled pH 5	++++	+++++	++++	++++	++++	+++++	+++	++	±	—
Antipneumococcus III horse serum absorbed	Pneumococcus III boiled pH 5	++++	+++++	++++	++++	++++	+++++	+++	+	±'	—
Antipneumococcus III horse serum unabsorbed	Pneumococcus III boiled pH 8.8	++++	+++++	++++	++++	++++	+++++	++++	++	±	—
Antipneumococcus III horse serum absorbed	Pneumococcus III boiled pH 8.8	++++	+++++	++++	++++	++++	+++++	++	+	±	—
Normal horse serum	Pneumococcus III formalinized	+++	++	±±	+	±					
Normal horse serum	Pneumococcus III boiled pH 5	+++±	++	±±	+	±					
Normal horse serum	Pneumococcus III boiled pH 8.8	+++	+++±	±±	+±	+					

Tubes in agglutination tests contained equal parts of diluted antiserum and antigen suspension. Readings after 16 hours in water bath at 50°C.

8.8, it fails to be agglutinated by the absorbed serum. That boiling at this reaction does not entirely destroy its capacity to act as an agglutinable particle in the presence of antiserum containing the S-precipitating antibody is also made clear.

Since, as is shown in Table III, the purified specific carbohydrates of the three pneumococcus types after boiling for 40 minutes at a reaction of pH 8.8 show no definite reduction of their function as precipitating agents, it is reasonable to infer that the constituent in Pneumococcus Type I responsible for the agglutination of the formalinized or acid-boiled organism in the absorbed antiserum and which is rendered inactive by boiling at pH 8.8 is a type-specific agglutinogen differing essentially from the specific carbohydrate.

Employing the same technique, the experiment was again performed using as antigens suspensions of Pneumococcus Type II. The results given in Table IV parallel those obtained for Pneumococcus Type I. Pneumococcus Type II also apparently contains an agglutinogen which is heat-labile at an alkaline reaction.

It will be noted that in the case of Pneumoccoccus Type I the titre of the unabsorbed antiserum was the same for all three suspensions. In the Type II unabsorbed rabbit antiserum the end-point of the titration in which the alkaline-boiled suspension is used falls short of that recorded for the formalinized and acid-boiled antigen. This fact affords further evidence of the presence of an agglutinogen distinct from the specific carbohydrate, since it is probable that the end-point of the alkaline-boiled suspension represents the final effect of the antibody reacting with the specific carbohydrate; while the end-points of the formalized and acid-boiled suspension indicate the quantity at which a second type-specific agglutinin ceases to react. A certain amount of experimental support is given to this hypothesis by the precipitating titre obtained by diluting the unabsorbed anti-Type II pneumococcus rabbit serum and using a constant quantity of the purified specific carbohydrate as antigen. The end-point of such a titration, to be found in Table IV, closely approximates that attained when the alkaline-boiled suspension is added to the same dilutions of the antiserum.

In parallel experiments with Pneumococcus Type III the outcome was somewhat different. The data assembled in Table V show that

TABLE V

Agglutination of Pneumococcus III in Unabsorbed and Absorbed Sera

Antiserum	Antigen	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1,280	1/2,560	1/5,120
Antipneumococcus III horse serum unabsorbed	Pneumococcus III formalinized	+++	+++	+++	+++	+++	+++	+++	++	±	—
Antipneumococcus III horse serum absorbed	Pneumococcus III formalinized	+++	+++	+++	+++	+++	++	+	—	—	—
Antipneumococcus III horse serum unabsorbed	Pneumococcus III boiled pH 5	+++	+++	+++	+++	+++	+++	+++	++	±	—
Antipneumococcus III horse serum absorbed	Pneumococcus III boiled pH 5	+++	+++	+++	+++	+++	+++	+++	+	±	—
Antipneumococcus III horse serum unabsorbed	Pneumococcus III boiled pH 8.8	+++	+++	+++	+++	+++	+++	+++	++	±	—
Antipneumococcus III horse serum absorbed	Pneumococcus III boiled pH 8.8	+++	+++	+++	+++	+++	+++	++	+	±	—
Normal horse serum	Pneumococcus III formalinized	+++	++	++	+	±					
Normal horse serum	Pneumococcus III boiled pH 5	+++±	++±	++	+	±					
Normal horse serum	Pneumococcus III boiled pH 8.8	+++	+++±	++±	++±	+					

Tubes in agglutination tests contained equal parts of diluted antiserum and antigen suspension. Readings after 16 hours in water bath at 50°C.

whereas this organism behaves like Pneumococcus Types I and II in being agglutinated to the same degree by unabsorbed and absorbed antisera, its susceptibility to agglutination in the absorbed antiserum is not removed by boiling at pH 8.8. Accordingly, it is impossible to assert with the same assurance that the agglutination of this organism in the absorbed antiserum is dependent upon an agglutinogen distinct from the specific carbohydrate. Nevertheless, it appears unlikely that the end-point of agglutination would remain practically unchanged after the removal from the antiserum of a very large proportion of the antibody against the specific carbohydrate if agglutination were dependent exclusively upon the interaction of those entities.

DISCUSSION

The experiments recorded above have shown that type-specific agglutination of Pneumococcus Types I, II and III occurs in homologous antiserum to approximately the same titre after the antibody reacting with the purified specific carbohydrate has been largely removed. This fact strongly suggests that in these organisms there exists a type-specific agglutinogen which is to be distinguished from the specific carbohydrate. The facts that boiling at pH 8.8 greatly reduces the agglutinability of Pneumococcus Type I and Type II in the presence of such absorbed sera, whereas boiling at pH 5 or formalinization leaves these organisms still susceptible to the agglutinating action of absorbed antisera reinforce the evidence for the existence of a type-specific agglutinogen other than the specific carbohydrate.

It has recently been shown (1) that the autolysate of Pneumococcus Type I contains a type-specific precipitating substance which is distinguished most markedly from the specific carbohydrate by its inability to resist heating in alkaline solution. Although the evidence is by no means sufficient to identify this heat-labile precipitating body or A substance with the type-specific agglutinogen described in this paper, the facts that they are both heat-labile in alkaline but not in acid solution and that both occur in antisera largely deprived of the antibody suggest that they may be the same antigenic substance.

Experimental support for a similar type-specific antigen in Pneumococcus Type III is not as adequate as in the cases of Pneumococcus I

and II. The fact, however, that antisera absorbed with the purified carbohydrate agglutinate the organism to the same titre as untreated sera points to the presence of an antigen of this nature.

Two points are perhaps worthy of comment in the light of the possibility of the existence of a type-specific agglutinogen in the pneumococcus unrelated to the specific carbohydrate. The first deals with the standardization of antisera for therapeutic use, such as antimeningococcus sera. At present the sole criterion for the therapeutic efficiency of antimeningococcus serum is a high agglutinin titre. Since it has been shown (2, 3) that at least as far as conferring protection goes in mice against the pneumococcus it is the specific carbohydrate antibody which is by far the most important if not the essential factor, and since these experiments make it clear that antisera may occur after immunization which contain no demonstrable precipitating antibody against that substance, but do contain agglutinins, it becomes evident that if standardization is based only on agglutinin titre, inadequate therapeusis may occasionally result.

The second application of the results of these experiments is to the possible explanation of the facts noted by Avery and his associates (4) that *B. friedlaenderi* Type B failed to absorb the agglutinins from Pneumococcus Type II antiserum, and that Pneumococcus Type II did not remove the agglutinins from *B. friedlaenderi* Type B antiserum, although these authors have shown that the specific carbohydrates derived from these organisms are probably identical.

The presence of a specific agglutinogen in Pneumococcus Type II unrelated to the specific carbohydrate would account for this failure to establish the complete serological identity of the two organisms.

CONCLUSIONS

1. Type-specific agglutination of Pneumococcus Types I, II and III has been demonstrated in antisera largely deprived of the carbohydrate antibody.

2. The type-specific agglutinogens in Pneumococcus I and II responsible for agglutination in such antisera are inactivated by heating in alkaline solution at 100°C. The specific carbohydrate remains unaltered under these conditions.

3. The relationship of the type-specific agglutinogen in Pneumococcus I to the type-specific A substance has been discussed.

4. The possible application of these results to the standardization of therapeutic antisera by agglutinin titre has been discussed.

5. On the basis of the experiments recorded a tentative explanation is offered for the failure of Pneumococcus Type II to absorb the agglutinins from anti-*B. friedlaenderi* Type B serum.

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THE RELATION OF HYDROSTATIC PRESSURE TO THE GRADIENT OF CAPILLARY PERMEABILITY

By PHILIP D. McMASTER, M.D., STEPHEN HUDACK, M.D., AND PEYTON ROUS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

PLATE 13

Despite the recognition in recent years of various physical and chemical factors which may conceivably influence exchange through the walls of the blood vessels, the view prevails that this is governed by diffusion, hydrostatic pressure, and the osmotic pressure of the blood colloids acting along capillaries which are equally permeable throughout their length. The demonstration that the escape of vital dyes from the blood into the tissues increases progressively along the capillaries of certain organs and is greatest precisely where it should be least if the factors just mentioned alone determined the phenomena (1) indicates either that some influence is at work which transcends these factors in importance, or that their rôle has not been wholly comprehended. That diffusibility directly conditions the distribution of dyes from the blood has been recognized since the work of Schulemann (2). The experiments here reported were undertaken to throw light on the influence of hydrostatic pressure. Our method has been, in brief, to modify this pressure markedly, inject a vital dye devoid of complicating affinities, and determine whether the changed conditions have altered the gradient of distribution from the capillaries.

General Technique

The ear of the mouse was used for some tests, but most were made upon the voluntary muscles of rabbits, because the arrangement of muscle capillaries is so regular that the local variations in opportunity for dyes to escape from the blood find expression in an almost diagrammatic color pattern. The dyes had been purified and tested for toxicity and complicating affinities (3). All were injected intrave-

nously in isotonic solution, as a rule in the amounts already found to be optimal for disclosure of the gradient of permeability. The escape from the blood was in some cases watched *in vivo* through mica windows let into the skin, and in others the tissue was examined immediately after the anesthetized animal had been exsanguinated from the carotids. Postmortem spread through the tissues is slow with poorly diffusible dyes like Chicago blue and pontamine sky blue, which were mainly employed. The technique of inspection has been described in previous articles.

Exclusion of the Factor of Specific Affinities

The vital dyes with which the work was done (Chicago blue 6B, pontamine sky blue, and brom phenol blue) are not fixed upon the tissues at once after they escape from the blood,—though later a portion of the two first mentioned is taken up and stored by some of the cells,—but they stain it because present in the interstitial fluid. By forcing this out the tissue can be decolorized. It follows that in determining the influence of hydrostatic pressure upon the escape of the dyes from the blood we are ascertaining how it affects the passage of the pigment from one fluid to another through the capillary membrane, that is to say are really dealing with capillary permeability.

Anesthetized rabbits, guinea pigs, and mice were injected with the dyes, and as soon as staining had become well marked the effect of pressure to drive out the color was tested. The ear of the mouse was frequently used. It was lopped off with scissors at the appropriate time and spread in oil between parallel glass plates, with a flexible sausage-shaped collodion bag about 2 mm. in diameter immediately under it. When the bag was filled with water from a manometer the narrow strip of tissue between it and the upper plate was pressed upon. In other instances the ear was placed between glass slides with a narrow strip of rubber underlying part of it, and the strip was repeatedly pressed against the ear, with some slight shifting, the object being to avoid any collection of dye into pockets in the compromised tissue. Considerable pressures could be exerted without deforming the ear.

The stained muscle (external oblique, gracilis of rabbits and guinea pigs) was excised as a sheet and spread and pressed upon in the same way, but with a bag about 6 mm. in diameter. Only slight pressures could be exerted since considerable ones squeezed the tissue thin, yielding a fallacious appearance of decolorization.

Both in the ear and in muscle the tissue pressed upon lost its color within a few minutes (Figs. 1 and 4). The dye-containing fluid that was forced out often formed

a more or less brilliant border to the decolorized strip. Chicago blue 6B, ponta-mine sky blue, brom phenol blue, trypan blue, red, and violet all yielded the same results. Since our object was to determine whether fixation upon the tissues acted to complicate the markings indicative of the gradient of vascular permeability, pressure was usually, though not always, brought to bear before these markings were obscured by diffuse coloration. Pressure regularly had the effect of obliter-ating them.

Effects of Nerve Section

It was necessary in many of the experiments to reduce the hydro-static pressure greatly. The fact was already known that when this is done by bleeding, the circulation to the viscera is maintained at the expense of that to the superficial tissues and voluntary muscles (4). If the depletion has been great, the blood flows through but a small proportion of the muscle vessels, and after dye injection its advancing, stained columns can be watched as they appear in the arteries, fork in the arterioles, and creep slowly along the capillaries. Despite the low blood pressure, the tissue coloration that gradually takes place has the pattern indicative of an unchanged gradient of vascular permeability, that is to say, the amount of dye escaping from the blood, as usual increases progressively along the capillary way (5). But the possibil-ity suggests itself that this may happen, not because of local differences in permeability but because of a tonic contraction of patent capilla-ries, greatest in their arterial portion and presumably affecting both the amount of wall through which dye can pass and the local per-meability. A less marked contraction of the same sort would account for the gradient of escape that is encountered normally. To test for the presence of such a state of affairs the muscle nerves were cut to. relax the vessels, and the distribution of dyes thereafter was ascer-tained. A leg was used, with its fellow as control.

To effect a wide-spread paralysis of the muscles of the hind leg of the rabbit it is necessary to cut the obturator and femoral nerves within the abdomen, and the sciatic high in the haunch. This was done under ether, with a minimum of trauma. Immediately afterwards a pronounced dilatation of the vessels could be discerned in the muscles studied,—gracilis, tibialis anticus, adductor longus,—a change espe-cially noticeable on comparison with the controls. Sometimes dye was injected into the blood stream at once, and, in other instances, from 4 to 24 hours after the nerve section, with renewed etherization. The legs were placed symmetrically and sometimes mica windows were inserted in the skin over the gracilis muscle. The carotid pressure was followed with a kymograph.

Staining of the paralyzed muscles took place far more rapidly than that of the controls and soon became general. For example, 10 minutes after injecting the ordinary amount of half strength Chicago blue (3 cc. of 8.5 per cent solution per kilo rabbit in 5 minutes), the muscles with cut nerves were deeply and evenly stained, whereas the controls showed the alternation of transverse blue and unstained bars ("mackerel sky" barring) characteristic of the gradient of permeability in long muscles. However, when the dye was injected very quickly and the animal was killed within 2 to 3 minutes the paralyzed muscle also showed these markings; but the blue bars were more intense and much broader than in the control tissue, a brilliant patterning. There was in addition a light general coloration that the control entirely lacked.

In some previous tests by one of us, carried out in the course of work with Dr. Gilding, highly diffusible dyes (patent blue V, brom phenol blue) were injected after nerve section; and a generalized staining of the paralyzed muscles was found at a time when the controls showed only a "mackerel sky" (6). This proved true even when blood bulk and pressure had been greatly reduced by bleeding. A repetition now with the same dyes, injected very quickly with immediate sacrifice of the animal, yielded a characteristic barring of the paralyzed muscles, the control tissue showing almost no color. This evidence of a persisting gradient of permeability had escaped attention before because it had been looked for too late, being already lost in a generalized staining.

The experiments make plain the fact that the gradient of capillary permeability is not the result of local contraction along the capillaries. It is true that when these vessels have been relaxed by nerve section dyes escape from them more rapidly than usual and the effective influence of the gradient of permeability extends over a larger proportion of the capillary; but the distribution is identical with that in normal muscle when a more diffusible dye has been employed, or a greater concentration of the one in present use. Increased blood flow past an increased amount of vascular wall,—which has doubtless undergone some thinning as result of the dilation,—will suffice to explain the findings.

Effects of Nerve Section and Reduced Hydrostatic Pressure

The next experiments were directed to reducing the hydrostatic pressure in paralyzed muscle to the minimum compatible with flow. Something had already been learnt on this point. For the rapid injection into animals with cut nerves of Chicago blue 6B had caused the pressure to fall to about half the normal height, yet the characteristic

banding developed. The tests now to be described were searching.

Under ether anesthesia the spinal cord of rabbits was cut in the upper lu region by a median laparotomy, or else between the third and fourth, or fourth fifth, thoracic vertebra by way of the back. The operation had the double e of greatly reducing the systemic blood pressure and of paralyzing many musc The best results for our purpose were obtained by a high transection, for then abdominal muscles,—which are especially suited to observations on the gradient permeability,—were paralyzed more than half way from the pelvis to the cost margin, a fact rendered evident, with the animal on its back, by a bulging above th pelvis and a relative constriction near the ribs where muscle tonus still existed.

To reduce the flow of blood to a minimum somewhere within the paralyzed ab- dominal muscle, the aorta was tied about 2 cm. below the left renal vessels. The median incision was closed with special care to prevent traction. Very soon after the ligation the skin of the lower part of the abdomen became pallid and cold, whereas in the region of paralysis further toward the ribs it was hyperemic owing to the vascular dilatation consequent on the cord transection (7).

Chicago blue was now injected, with the animal on its back, and a few minutes later it was bled to death from the carotids. Staining of the skin of the upper abdomen had already developed, extending about midway to the groins. The external oblique muscle proved to be completely unstained in the groin region, that is to say in the lower part of its paralyzed portion; and no circulation could have existed here for the residual blood was not stained in the least. Further toward the ribs the arteries showed dye-stained blood, but not the veins; a little further on both contained it, and there was a definite, if pale, extravascular coloration having the fern pattern referable to the gradient in short muscles (8); while beyond, in the region where the aortic ligation had not interfered with circulation, this pattern was pronounced. Here an unusual amount of blood remained in the small vessels. The zone of transition from unstained to deeply stained tissue was only about 1 cm. wide (Figs. 2 and 3).

After low cord transection and aortic ligation, a feeble circulation sometimes took place through the paralyzed gracilis, this muscle staining very slowly and irregularly. But where it stained at all, no matter how palely, "mackerel sky" bands, attesting to the persistence of the gradient of permeability, could be per- ceived.

In the animals of these experiments, subjected to cord transection and aortic ligation, dye escaped through the capillary wall into the tissue wherever the blood pressure was sufficient to move the blood along the relaxed vessels of the paralyzed muscle. The staining that developed in such regions showed that the gradient of capillary per- meability had undergone no alteration as result of the muscle paralysis

and the minimal blood pressure (Figs. 2 and 3). It cannot immediately be concluded, however, that the gradient of permeability is independent of hydrostatic conditions. The venous system was intact; and pressure in the large veins is known to be almost independent of that in the arteries (9). The gradual increase in staining that took place along the capillaries as the venules were neared, though resembling that due to the ordinary gradient of capillary permeability, might conceivably have resulted from the influence of a venous pressure of normal height in association with an arterial pressure which had been diminished to the limit compatible with flow. In work to be published separately we have found that a slight increase in venous pressure over the normal emphasizes the gradient of permeability along the cutaneous vessels, even more dye than ordinary getting out from the further part of the capillaries and from the venules,—which in the skin have ordinarily a permeability transcending that of the capillaries. Chicago blue does not escape from the venules of muscle under ordinary conditions, nor did it in the experiments just described, as could plainly be seen when a sheet of the external oblique was dissected away and studied under the binocular microscope in the usual combination of transmitted and reflected light (10). But under the circumstances of increased venous pressure an escape does take place, as will be shown further on.

To test the possibility just outlined, high cord transection and aortic ligation were done in some further experiments, and by means of two wire snares the vena cava was occluded below the renal vessels and severed further down, just before a dye was placed in circulation. Since the blood from the portion of the abdominal muscles below the level of the umbilicus reaches the cava by tributaries which enter not more than 1 cm. above the junction of the iliacs, we were able to exclude the systemic venous pressure as a possible factor in the results by opening the cava further up. One experiment will be described in detail as representative of both the procedure and the findings.

A white male rabbit of 1790 gm. was etherized and shaved at 3:40 p.m., about 5 hours after feeding. The spinal cord was transected through an incision in the back between the fourth and fifth thoracic vertebrae, the abdomen was opened in the midline, the renal veins tied, and two snares placed around the vena cava, one just above the right adrenal gland, the other well below the left renal vein. The higher

of the two consisted of a loop of stout thread projecting from the end of a glass tube which had been pinched together in the flame to close it, save for two small openings through which the thread passed. Pulling upon the free ends of the thread obstructed the cava by drawing it against the tube. The other snare consisted of a loop of fine copper wire, instead of thread, and the end of the glass tube had sharp edges. After they had been put in position the snares were left loose about the vein, with the tubes,—which were narrow, and so thin-walled as to be very light,—projecting through the abdominal incision. This was closed around them after ligation of the aorta 2 cm. below the left renal vein. All had been accomplished by 4:30 p.m. At that time the abdomen presented a curious spectacle. Despite a stomach crammed with food its upper two-fifths appeared slender by contrast with a broad pouching of the lower portion, consequent upon a flaccid paralysis of the lower abdominal wall. All of the intestines lay in the pouch, and when pushed toward the diaphragm by manual pressure they slid back into it again. The skin of the upper part of the abdomen showed many abnormally distended venules carrying bright blood. Its pouched lower half and the hind legs were cold and pallid, although the animal, in excellent general condition, lay on a warm electric pad.

At 4:32 p.m. a carotid artery was cannulated and a kymograph tracing started. The pressure proved steady, at 26 mm. Hg.

4:41 p.m. The upper snare was severed by pulling upon the wire of the lower snare. once thereafter this vessel was drawn upon to occlude the vena cava, and at The injection was begun forthwith of 6 cc. of a warmed isotonic solution of half strength Chicago blue through a needle already placed in an ear vein. It was completed in 5 minutes and 5 seconds. At the end of the first 2 minutes the blood coursing in the cutaneous veins of the upper abdomen had become blue; after another half minute staining in this region had begun; and when the carotids were cut 1½ minutes after the injection was finished the skin was blue halfway from ribs to pelvis. Further down its bloodless, pallid appearance was unchanged. The carotid pressure was 27 mm. Hg at the beginning of the injection; at its end, 24 mm., not varying thereafter. On stripping back the skin immediately after death, fern-like blue markings were to be seen in the muscles of the abdominal wall, but no generalized staining of them. The markings extended somewhat more than halfway toward the pelvis, fading off rather abruptly into unstained tissue further down The external oblique muscle was dissected away in two large sheets which included the zone of transition from staining to pallor. Under a magnifying glass the fact could be made out that wherever stained blood had got through to the veins in quantity a fern-like blue coloration of the tissue had taken place, such as is the result of the gradient of capillary permeability. At the far edge of the zone of transition from stained to unstained tissue only the arterial blood was deeply colored; and here no perceptible dye had got out into the muscle. Where the staining of this tissue was palest the local differences in it could yet be well seen, much as the pattern of the feathers of a white peacock is seen; and the width of the individual bands of blue appeared to be but little less than where the

coloration was intense. The vessels of the muscle appeared broader and contained more residual blood than usual. There was no diffuse staining whatever. The findings were identical with those when the vena cava was intact.

From the torn cava only 2.5 cc. of blood had been lost, very moderately colored with dye, as would follow from the circumstance that it was derived from a region in which the circulation had been greatly interfered with.

The distribution of dye from the capillaries after the vena cava had been severed did not differ from that when this vessel was intact. It follows that transmission backwards of the systemic venous pressure can be excluded as the cause for the gradient of vascular permeability observed in paralyzed muscle through which blood is coursing at the minimum pressure compatible with flow. One must conclude that this gradient exists independently of local variations in the hydrostatic pressure. This is not to say that it may not be modified by pressure differences. In rabbits with a normal arterial pressure a generalized staining of paralyzed leg muscles was found to take place concomitantly with the more intense local one referable to the gradient. There had manifestly taken place an escape of dye from the first portion of the capillaries as well as further on. Whether this escape was due to the increase in permeable wall surface resulting from dilatation of the capillaries after nerve section, or to transmission of the arterial pressure into their first portion, or to both causes, was not certain from the experiments disclosing it. But observations after cord transection and aortic ligation settled the point. In such instances the quantity of dye carried by the blood was often considerably greater than usual, owing both to a purposeful increase in the amount injected (sometimes double the usual dose) and to a reduction in the volume of blood in which it circulated, consequent upon a locking up of a portion in the parts affected by the ligation; yet no general staining from the dilated capillaries of that part of the paralyzed muscle in which circulation was good accompanied the local one from their further portion which was referable to the gradient. It is plain that capillary dilatation after nerve section does not inevitably result in a generalized escape of dye. The animals in which it was lacking had a greatly reduced systemic blood pressure. That this was the reason for the absence of generalized staining seems the more likely because of experiments now to be

described in which a heightened venous pressure resulted in an increased local escape of dye from the vessels.

Effects of Altering the Venous Pressure

For excellent reasons the results of increasing the hydrostatic pressure were ascertained by raising the venous pressure instead of the arterial. The permeability of the capillaries is greatest at their venous ends, and one would expect alterations in dye distribution as the result of pressure changes to be most pronounced there. Furthermore the venous pressure could readily be quadrupled, whereas the arterial could be raised only fractionally and by a contraction of the arteries themselves.

It was not feasible to make the tests on muscles with cut nerves because of the rapidity with which dye escapes from the dilated vessels when the blood pressure is merely normal. But clear-cut indications were obtained in intact preparations that raising the venous pressure causes an increased escape of dye through the capillary wall.

The saphenous and femoral veins of a hind leg of rabbits under ether or luminal anesthesia were tied, and, as control, the same veins on the other side were dissected out, but without ligation. The legs were then placed symmetrically, with the animal on its back; and the dye was injected into an ear vein as ordinarily. Chicago blue was used for the most part but sometimes brom phenol blue, and in one instance phenol red. After the dye had been in circulation for a few minutes the carotids were cut and the tibialis anticus muscles were compared. It was regularly found that the tissue on the side of venous obstruction showed broader and deeper bars of stain. After phenol red there was a generalized staining also, as would follow from its high diffusibility (11).

To produce a greater degree of obstruction in some cases the common iliac, popliteal, and saphenous veins were tied on one side while on the other they were merely dissected free. The findings did not differ from those just described.

Obstruction of the femoral vein had no effect whatever to cause venous congestion, owing to the abundance of collaterals. Because of these the vessel could be utilized for determinations of the venous pressure in the leg after obstruction of the other veins draining it. In numerous animals the distal end of each femoral was connected with a salt solution manometer after heparin had been injected into the blood stream to prevent clotting. The pressures proved to be approximately the same on both sides, from 5 to 6.5 cm. of salt solution. Now one common iliac vein was tied off through an abdominal incision and the other merely dissected free. This resulted within a few minutes in a rise of the femoral pressure on the side of the ligation to 22 to 26 cm. while on the other side the pressure did not change.

Dye was injected. It appeared promptly in the muscle vessels exposed under a mica window, showing that arterial blood was still entering them despite the accumulation consequent on the iliac tie. Exsanguination was done a few minutes later, both from the carotids and the femoral veins; and several muscles from each lower leg were removed and compared. The staining took the usual banded form, but the bands were broader and deeper colored where there had been increased venous pressure. More dye had escaped from the capillaries on this side and further back along them.

In three animals the superior mammary and the superficial and deep epigastric veins of one side were ligated, and 2 to 6 hours later Chicago blue was placed in circulation. Through mica windows overlying the muscle the fact could be made out that the dye reached the venules of the external oblique more slowly on the side of the ligations. Nevertheless when the animals were killed, after a few minutes, the barring was, in two of three instances, broader and deeper than in the control, and in one case the dye had escaped from the transverse venules as well, forming a blue zone along them.

The vena cava of one rabbit was ligated just above the junction of the common iliac veins and 82 minutes later Chicago blue was injected. The staining of the abdominal muscle proved to be more intense in the region draining into the vena cava below the tie; and in the hind legs the barring was darker and the general staining deeper than in the pectorals and fore legs. In another animal 2 days were allowed to elapse after operation before the dye was given,—during which period no limp or paralysis was observed and a well defined collateral circulation formed by way of the inferior and superior epigastric veins. In the muscle regions affected by the obstruction, the same local intensification of the staining was noted as in the other experiments, and dye escaped from the transverse venules as well as from the capillaries. Like results were obtained 3 days after the vena cava had been tied off above the inferior mesenteric vein. Just prior to the injection of dye into the ear the femoral veins were cannulated and abnormally high pressures found in them—26 to 27 cm. in the distal ends in the case of one rabbit, in another 10 to 13 cm. in the proximal ends.

When only one common iliac had been tied so effective a collateral circulation developed within 3 or 4 days that the pressure in the distal end of the femoral vein on the obstructed side was but slightly higher than on the other. Dye injection yielded a better staining on the free side than on the obstructed.

Raising the venous pressure markedly by obstructing outflow enhanced the escape of dye put in circulation shortly afterwards and increased the proportion of capillary from which it took place. This was clear from the character of the staining. The bands of color were broader and deeper than ordinary and extended further back along the capillaries. When the dye was placed in circulation after obstruction had endured several days the findings varied with whether

collaterals had relieved the abnormal pressure condition or not. When they had done so, as after ligation of a common iliac vein, the staining was not increased on the obstructed side and was often less than on the other. But when the venous pressure was still notably high, as when the vena cava had been tied, not only did more dye escape and the effective permeability extend further back along the capillary but the venules proved permeable to the dye, as happened only exceptionally when the pressure rise had been of short duration.

Flow through the muscles subjected to partial venous obstruction was retarded, as could be told by the rate of passage of the dye-stained blood into the small vessels. There was, of course, an accumulation back of the obstruction and in addition, doubtless, some induced contraction of the artery feeding the part (12). The turnover of blood cannot but have been less than in the control limb. If the substances employed had been so diffusible as to escape readily into the tissue, one might have expected the lessened quantity that was available on the experimental side to be reflected in a lighter staining, despite the heightened venous pressure, since the depth of the staining would be an immediate expression of the amount of dye brought by the blood. This proved to be the actual case in experiments with brom phenol blue. But Chicago blue is so poorly diffusible that only a very small part of the amount passing through the muscle at a given moment escapes into the tissue. Our results show that the rapid replacement of the dye-laden blood with more of the same sort, as when flow is active, has less importance for the distribution of Chicago blue to the tissues than the influence of increased venous pressure. Otherwise the staining could not have been heavier in the leg subject to passive hyperemia than it was in the control

The possibility must be considered that the heightened venous pressure resulted in an increased escape from the capillaries because some of those through which no circulation had been taking place were forced open by it,—not because the permeability of the individual vessels was increased. But this would not suffice to explain the increased permeability of the venules. That even very high pressures fail to open closed muscle capillaries is a truism with those who have attempted an injection of colored mass to demonstrate the vascular arrangement.

The effects of increasing the venous pressure were so marked as to suggest the possibility that the normal blood pressure at the venous end of the capillary may be at least partially responsible for the relatively abundant escape of dyes into the tissue at this situation,—though true it is that the experiments in which the vena cava had been severed after aortic ligation yielded no indications of the sort. To settle the point a procedure was devised whereby the venous pressure could at will be reduced to zero without any loss of blood.

Rabbits of approximately 2000 gm. were closely paired by weight after the withdrawal of food for 24 hours. Cross-agglutination tests had shown their bloods to be compatible. They were anesthetized with a 10 per cent solution of sodium amytal (0.5 cc. per kilo intravenously, with 0.25 cc. half an hour afterwards, and more later if necessary), and laid upon warmed pads, side by side on their backs with the hind legs placed symmetrically. The proximal ends of one common carotid of each animal were connected by a rubber tube having a glass T inserted midway, the stem of which led to a mercury manometer. The tubes had been filled with Ringer's solution prior to insertion. The distal end of one common iliac vein of the rabbit with the lower carotid pressure was then connected with the proximal end of the opposite vein of the other animal, by means of wide-mouthed cannulae and a rubber tube having a larger bore than that of the veins, which connected through a T with a manometer containing Ringer's solution. The uncannulated common iliacs were dissected free as a control to the effects of trauma, and fine threads were put loosely about them so that they could be lifted and cut at will. The abdominal incisions were closed about the tube connecting the veins and 1½ cc. per kilo of a solution containing 10 mg. of heparin per cc. was injected intravenously into each animal to prevent clotting when the connections were opened.

No nerves were severed, since our aim was to determine the effects of abolishing the venous pressure in intact muscles. It was necessary, however, to clamp off the common iliac vein for a few minutes during the cannulation.

The arterial and venous connections were now opened, and at intervals the individual pressures in each animal were taken by temporarily clamping the rubber tube to one side or other of the manometers. Flow from the distal end of the common iliac of the upper animal was not blocked long enough in this way ordinarily for a reading to be obtained of the highest pressure developing on obstruction, but only momentarily so that one could learn from the prompt rise in the manometer column that active flow had been going on. The development of a negative pressure when the salt solution manometer was raised above its previous level constituted another check upon the existence of flow. Now the rabbit receiving venous blood was gradually lowered so that it lay 10 to 20 cm. below the other. After the lapse of a few minutes to allow for circulatory readjustments,— which took place without any change in the mean carotid pressure,—individual

readings were taken again, with the manometers at the levels of the upper and lower animals respectively, and dye was injected simultaneously into one or both of them. They were killed soon after by cutting the carotids and all four common iliac veins.

Six technically adequate experiments were accomplished, three with Chicago blue, three with brom phenol blue. The animals remained in excellent condition throughout, because care had been taken to keep them warm and to minimize trauma. Except during the determinations of pressure, all of the connections were left open, the mean pressure in the connections being followed as indices to the general state of affairs. The mean pressure in the arterial connection, while varying little in the individual experiment, ranged from 70 to 95 mm. Hg for the entire group. The pressure in the proximal end of the common iliac of the lower rabbit varied between 5 and 7 cm. of 0.9 per cent salt solution when flow into this vessel from the other rabbit was cut off, a figure corresponding with that obtained by cannulating the proximal end of a femoral vein under more ordinary circumstances, as in the experiments on venous obstruction already described. The possibility of a positive pressure in the distal portion of the common iliac of the upper rabbit was ruled out by manometer readings which showed the existence of a negative pull when the instrument was raised to the level of this animal. The injection of Chicago blue into the upper animal caused a transient fall in its carotid pressure, which was reflected in some lessening of the mean carotid pressure; and through the walls of the glass T connecting the iliac veins the prompt passage af the dye-stained blood to the lower rabbit could be observed, though it never took place in sufficient amount for a noticeable staining of the latter. Sometimes this animal was preserved for a few minutes after sacrifice of the other, to be utilized for a separate test of the effect on the gradient of permeability of ligation of the common iliac vein.

The corresponding muscles of the hind legs of the upper animal were compared both *in situ* and after excision. The tibialis anticus and adductor longus were principally scrutinized.

In these cross-transfusion experiments the lowering of the venous pressure to zero may have brought about local changes by way of the vasomotor system. However this may be, the results in terms of dye distribution were precisely those that would have been expected from the fact that raising the pressure emphasized the gradient of permeability and extend its effective scope. The staining with Chicago blue was slightly but definitely less intense than in the control muscles, and the bars of color were a little narrower. With brom phenol blue no differences could be perceived. As already mentioned, staining with this dye is immediately dependent on the amount of stained blood that circulates through the tissue. The influence of this factor may well have masked that of the differing venous pressures.

Pressure importantly conditions the gradient however. Raising the venous pressure extends the effective scope of the gradient and accentuates the local differences in permeability. In whatever way this is accomplished, whether by a stretching and thinning of the vessel, by a filtration process, or by both, accomplished it assuredly is. Landis' direct measurements prove that an increase in venous pressure causes a prompt rise of that within the capillaries of human skin (17). In some of our experiments the positive pressure in the large veins draining the muscles was reduced to zero. Needless to say the pressure in the capillaries,—which are long and very slender,—may not have been profoundly lowered under such circumstances; for it must be largely dependent on the driving force of the blood. All one could hope to produce by the change was a greater pressure fall than ordinary along the capillary way, a fall which might possibly become manifest in an alteration in the rate and character of the distribution of the dyes. Alterations did take place, such as might be taken to indicate that even normal venous pressure is not without a favoring influence on the escape of substances into the tissues from the further capillary region. But induced vasomotor reactions cannot be ruled out as responsible for the changes.

Substances of small molecule leave the blood with such rapidity by diffusion that their spread may be considered as largely independent of any filtration of water (18). Even with dyes of large molecule, diffusion is an important influence in distribution, the rate of vital staining varying directly with the diffusibility of the pigment as ascertained *in vitro* (19). The escape of Chicago blue, after arrest of the circulation and exclusion of the pressure factor, must be laid to diffusion. According to Landis the rate of water filtration, not the intrinsic permeability of the capillaries, is indicated by the spread of dyes (20). Such was doubtless the case with the material, and under the circumstances, of his experiments, which involved long exposure and irrigation of the mesentery of the frog; but a repetition of his tests under controlled conditions has proved their results due to vascular disturbance (21). If the coloration of resting mammalian muscle, an organ from which there is practically no lymph flow (22), were due to a filtration of dye-stained water one would have to suppose a closed circulation of such water in the region supplied by the capillary, occurring pre-

sumably by the influence of the hydrostatic pressure to force water out through the wall of the first portion of the vessel and the osmotic influence of the blood colloids, further on, where pressure had fallen, to bring it in again (23). The mounting gradient of distribution of dyes along the capillary is incompatible with this filtration hypothesis (24), and the persistance of the gradient when the hydrostatic pressure is greatly reduced, or done away with, effectually disposes of it.

The dyes were in watery solution and where water goes they may be supposed to go, unless their molecules are large enough to be held back selectively. When the arterial pressure is markedly lowered, as was the case in many of our experiments, water enters the blood from the tissues; but so swift is the process of readjustment (25) that it was doubtless at an end before our dyes were placed in circulation. Whether a fluid transfer took place in the experiments in which the venous pressure was raised present findings do not enable us to say. In a succeeding paper, however, the influence of water flow, and of the blood colloids, on the gradient of vascular permeability will be dealt with directly.

None of the highly various functional disturbances of the vessels and circulation brought about in the present work and that previously reported has abolished the gradient of vascular permeability. The local changes incidental to plethora, hemorrhage, shock, nerve section, active and passive hyperemia, muscular contraction, circulatory arrest, and anhydremia due to hypertonic salt solution administered by mouth, have alike proved ineffectual in this respect. Consequently it is difficult to suppose that the gradient can be the outcome of functional conditions. Rather should its cause be sought in a structural differentiation along the capillary.

SUMMARY

The gradient of permeability along the capillaries of voluntary muscle and the capillaries and venules of skin exists independently of the hydrostatic conditions, though influenced by them. Its presence cannot be explained by a graded tonic contraction of the capillaries. The evidence,—like that of previous papers,—points to local differences in the barrier offered by the wall of these vessels as responsible for the gradient.

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EXPLANATION OF PLATE 13

FIG. 1. Decolorization by pressure of diffusely stained tissue. Portion of an external oblique muscle of a rabbit, upon which pressure was brought to bear by means of a bag lying beneath the preparation and separated from it by a sheet of moist, white paper. The dye forced from the tissue has accumulated to either side of the strip pressed upon. In some regions the pressure was insufficient to empty the large vessels entirely. Photographed by reflected light. ×8½.

FIG. 2. Part of an external oblique muscle of a rabbit receiving Chicago blue 6B immediately after the spinal cord had been cut between the fourth and fifth thoracic vertebrae, and the aorta tied below the renal vessels. The dye injection

took 5 minutes and the animal was bled to death 2 minutes later. The sheet of muscle was excised immediately and photographed through a red filter to exclude the blood color. The specimen includes the zone of transition from stained to unstained tissue. It will be seen that wherever dye has passed through the vessels in quantity, as shown by the dark contents of the veins, a staining indicative of the ordinary gradient of vascular permeability has developed. Natural size.

Fig. 3. A portion of the transition zone of Fig. 2, enlarged 2 times.

Fig. 4. Decoloration by pressure of tissue showing the markings indicative of the gradient of vascular permeability. The ear of a mouse injected with pontamine blue was lopped off early in the period of dye escape, when the local differences in staining were well defined; and a strip of rubber was pressed upon the organ in the region X, according to the method described in the text. The picture shows that the tissue pressed upon was decolorized. The dye-stained fluid deriving from it forms a dark border on the side toward the base of the ear. × 3.

PLATE 13

(McMaster *et al.*: Gradient of capillary permeability)
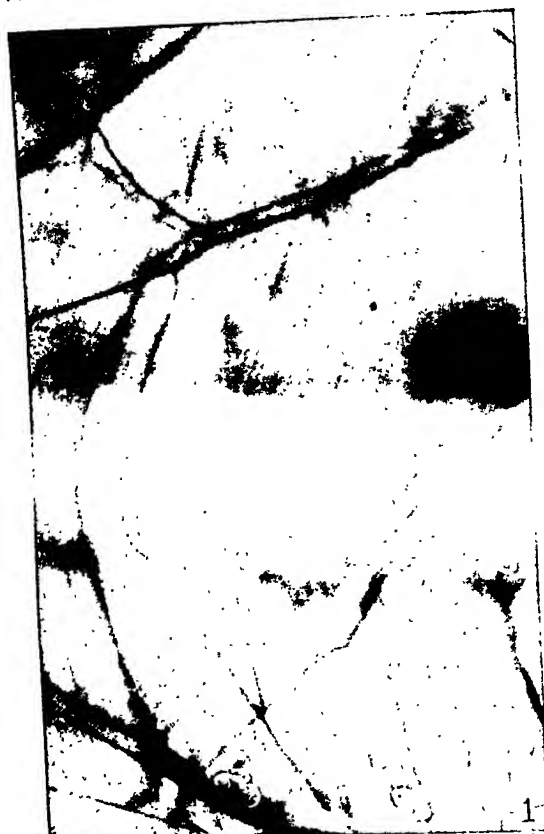
STUDIES ON INFLUENZAL MENINGITIS

I. The Problems of Specific Therapy*

By HUGH K. WARD, M.B., and JOYCE WRIGHT, B.M., B.Ch.

(From the Department of Bacteriology and Immunology of the Harvard University Medical School, Boston)

INTRODUCTION

Whatever the relation of Pfeiffer's *Bacillus influenzae* to epidemic influenza—and that is not under discussion here—there is no doubt that this organism is the cause of influenzal meningitis, a disease that is much more common than is generally realized. The records of The Children's Hospital, Boston, disclose that in the last 5 years there have been 56 cases of meningococcus meningitis and 25 cases of influenzal meningitis in children under 2 years of age. In both diseases the cases have occurred only sporadically during this period.

Influenzal meningitis is a disease of infancy and early childhood and is almost always fatal. Rivers (1), in an analysis from the literature of 220 cases under 2 years of age, gives a mortality of 92 per cent. It is remarkable that no record can be found of any attempt being made to treat these cases with an appropriate antiserum, despite the striking results that have been obtained with antimeningococcus serum. Wollstein (2) in 1911 injected antiserum intraspinally 24 hours after she had infected a *rhesus* monkey, the animal recovering, but no account can be found of the use of this serum in human cases. Rivers (3) has pointed out, and this has been our own experience, that the strains of the influenza bacillus that are found in influenzal meningitis are fairly homogeneous, thus simplifying the problem of specific serum treatment. Under these circumstances we thought it worth while to investigate the possibility of specific therapy. Through the courtesy of the Massachusetts Antitoxin Laboratory, after some months we

had available for use the serum of a horse that had been injected with formalized cultures of *B. influenzae* that had been recently isolated from cases of the disease. This antiserum was tried on a number of cases, some 10 to 12 cc. being injected intraspinally either once or twice a day. Although the serum agglutinated the organisms isolated from the cases and was therefore apparently specific, it had no effect whatever on the course of the disease, and influenza bacilli could be cultured from every specimen of cerebrospinal fluid up to the day of death.

The disappointing clinical results with this antiserum and the information that it contained no precipitins[1] prompted us to make a more thorough investigation into the problem instead of merely treating the patients empirically with a specifically agglutinating antiserum. How was this investigation to be carried out? Direct animal experimentation was not promising, because none of the ordinary laboratory animals are susceptible to virulent *B. influenzae* except in large doses and there is very little margin between the lethal dose of living organisms and the lethal dose of dead organisms. This suggests that the toxicity of the culture is of much greater importance than the virulence in overcoming the resistance of the animals and in the beginning at any rate it was the virulence-resistance mechanism rather than the toxicity of the organism that we wished to study.

In the case of the pneumococcus, bactericidal tests either with whole blood or mixtures of leucocytes and serum have given useful information in the problems of pneumonia, several workers, including one of the authors, having employed this technique in recent years. With this experience as a general guide, it was decided to use a similar method in influenzal meningitis, but one modified to meet the special characteristics of the influenza bacillus. It is admitted that, in attempting to find out what goes on in the body in infectious diseases by studying the behaviour of the specific virus in the blood only, one has to be very cautious in drawing inferences. For although the serum and leucocytes may be the most important part of the defense mechanism, they are still only a part. Nevertheless, the technique of the bactericidal blood test is so easy and simple compared to *in vivo* tests

[1] We owe this knowledge to Dr. Margaret Pittman of The Rockefeller Institute who tested the serum and kindly passed on the information to us.

that it facilitates the study of the practical problems of specific therapy, as well as the study of the relationship between defences of the parasite (upon which virulence depends) and the defences of the host (upon which resistance depends). And in diseases in which there is no susceptible animal available, it seemed to us the only way at present to study these problems.

Technique

The technique used in these experiments is essentially one devised originally by Todd, but slightly modified by one of the authors and described in previous publications (4). One determines the maximum number of organisms that a constant amount of the whole defibrinated blood will kill, in the following manner:

Known but varying numbers of organisms are incubated for 24 hours with the constant amount of blood (0.5 cc.) in small, sealed test-tubes placed in a rotating box. The tubes are then broken open and the contents plated out to determine growth or sterility. Growth products of the organisms or antibodies or both can be added to the contents of the tubes before incubation if one wishes to study the effect of these substances on the bactericidal action of the blood. Details of technique can be found in the original communication (4).

In working with B. influenzae, certain minor changes have been made:

1. In experiments involving phagocytosis, human defibrinated whole blood has been used, because rabbit blood is not suitable for this purpose, but in all other experiments rabbit defibrinated whole blood has been employed. Fresh rabbit serum has been tried occasionally, but the results are the same as with the whole blood, and it is more convenient to use the blood. In some experiments where it was desirable to reduce the bactericidal power of the normal blood in order to show the effect of adding antibody, the blood has been diluted with an equal volume of 5 per cent pepsin-digested horse blood broth. There is still enough complement left in this special blood broth mixture to show bactericidal action in the presence of antibody, and this digested blood broth is an excellent medium for the B. influenzae. Fildes (5) advocated the use of this broth and described the method of preparation in 1920.

2. The smooth, virulent cultures of B. influenzae have been kept in defibrinated rabbit blood for the greater part of the time. As far as we can tell, this method has been successful in maintaining the original virulence of the cultures, a very important consideration in most bacteriological work, and especially in studies of this nature. The organism growing out from the culture of cerebrospinal fluid is inoculated into about 0.5 cc. of fresh, defibrinated rabbit blood in either a sealed or a wool-plugged tube. The tube is incubated for 24 hours and then placed in the ice box. At the end of 10 days a tube of 5 per cent digested blood broth is inoculated from the blood tube. The broth culture is incubated and the process repeated.

The rough, non-virulent cultures of *B. influenzae* have been kept in the 5 per cent digested blood broth in the ice box, transplanting at 10 day intervals.

3. When a culture of the virulent organism is wanted for the bactericidal blood test, an 18 hour culture is made in the 5 per cent digested blood broth from one of the stock blood tubes described above.

4. At the end of the incubation of the blood tubes in the bactericidal test, the tubes are broken open and their contents plated out with a platinum loop on to chocolate agar instead of blood agar.

EXPERIMENTAL

In any test involving the bactericidal action of the blood on a certain species of organism, it is obviously necessary to begin by analysing this action. Generally speaking, the Gram-positive organisms are first sensitized by the antibody in the serum and then phagocyted by the leucocytes, no organisms being killed in the absence of the cells; but with the Gram-negative organisms, the cells play a very minor rôle, the bacteria being sensitized by the antibody and then killed by the complement. Under certain conditions the sensitized organisms are not only killed, but undergo lysis. *B. influenzae* falls into this category, but it was found that the cerebrospinal fluid in cases of influenzal meningitis never contained a trace of complement, but leucocytes were always present, often in great numbers. Under these circumstances it was desirable to find out whether phagocytosis of the bacilli could occur in the presence of antibody and cells, but in the absence of complement.

Table I shows the results of an experiment designed to demonstrate the bactericidal action of the various constituents of normal human blood against a virulent culture of *B. influenzae*. The blood was separated into serum and cells by centrifugation. Part of the serum was heated to 56°C. for 30 minutes. The cells were washed four times with saline before using. Antiserum in various concentrations was added to the cells, because the cells with saline or with heated serum had no bactericidal action at all.

Table I shows:

1. That 1.0 cc. of normal human blood can kill at least 10,000,000 virulent influenza bacilli, and it may be asked how does this very strong bactericidal power of the blood fit in with the known susceptibility of human beings to influenzal meningitis, if such findings are at all typical. As far as we know at present, this result is typical of

adult blood, but not of infant blood, and influenzal meningitis is a
disease of infancy. This, however, is a subject for separate in-
vestigation.

2. That the action of the unheated normal serum alone is just as
strong as that of the whole blood, and that the cells either with saline
or heated normal serum have no action at all. In other words, the
bactericidal action of normal whole blood against *B. influenzae* is
wholly due to the natural antibody-complement mechanism.

3. That the organism does not grow well in heated normal serum
alone, but that there can be no bactericidal action, as is shown by the

TABLE I

No. of organisms added	Whole blood	Unheated serum	Heated serum	Washed cells + saline	Washed cells + heated serum	Washed cells + heated serum + concentration of antiserum		
						1/20	1/200	1/2,000
5,000,000	0	0	++	++++	++++	++++	++++	++++
500,000	0	0	++	++++	++++	++++	++++	++++
50,000	0	0	++	++++	++++	++++	+++	++++
5,000	0	0	++	++++	++++	+++	+++	++
500	0	0	+	++++	++++	++	+	+
50	0	0	+	++++	++++	0	+	0
5	0	0	0	++++	++++	0	0	0

Here, and in the following tables, ++++, +++, ++, + = various degrees
of growth. 0 sterility.

addition of cells to the heated serum, the cells probably supplying a
necessary growth factor.

4. That the addition of antiserum to the cells and heated serum re-
sults in a very slight bactericidal action presumably by phagocytosis.

Kolmer (6) pointed out several years ago that complement was ab-
sent from the cerebrospinal fluid of cases of influenzal meningitis,
and this finding has been confirmed in the present investigation. It is
interesting in this connection that Dr. L. D. Fothergill of The Chil-
dren's Hospital, Boston, has shown the presence of complement in the
cerebrospinal fluid of all cases of meningococcus meningitis that he has
tested. The method of carrying out the test for complement in the
two diseases was identical.

The rough, non-virulent cultures of *B. influenzae* have been kept in the 5 per cent digested blood broth in the ice box, transplanting at 10 day intervals.

3. When a culture of the virulent organism is wanted for the bactericidal blood test, an 18 hour culture is made in the 5 per cent digested blood broth from one of the stock blood tubes described above.

4. At the end of the incubation of the blood tubes in the bactericidal test, the tubes are broken open and their contents plated out with a platinum loop on to chocolate agar instead of blood agar.

EXPERIMENTAL

In any test involving the bactericidal action of the blood on a certain species of organism, it is obviously necessary to begin by analysing this action.   Generally speaking, the Gram-positive organisms are first sensitized by the antibody in the serum and then phagocyted by the leucocytes, no organisms being killed in the absence of the cells; but with the Gram-negative organisms, the cells play a very minor rôle, the bacteria being sensitized by the antibody and then killed by the complement.   Under certain conditions the sensitized organisms are not only killed, but undergo lysis.   *B. influenzae* falls into this category, but it was found that the cerebrospinal fluid in cases of influenzal meningitis never contained a trace of complement, but leucocytes were always present, often in great numbers.   Under these circumstances it was desirable to find out whether phagocytosis of the bacilli could occur in the presence of antibody and cells, but in the absence of complement.

Table I shows the results of an experiment designed to demonstrate the bactericidal action of the various constituents of normal human blood against a virulent culture of *B. influenzae*.   The blood was separated into serum and cells by centrifugation.   Part of the serum was heated to 56°C. for 30 minutes.   The cells were washed four times with saline before using.   Antiserum in various concentrations was added to the cells, because the cells with saline or with heated serum had no bactericidal action at all.

Table I shows:

1. That 1.0 cc. of normal human blood can kill at least 10,000,000 virulent influenza bacilli, and it may be asked how does this very strong bactericidal power of the blood fit in with the known susceptibility of human beings to influenzal meningitis, if such findings are at all typical.   As far as we know at present, this result is typical of

complement as efficient as possible, since it is logical to assume that the more rapidly the meninges are sterilized, the less likely is the formation of abscesses.

In the case of antimeningococcus serum, in which no animal test is available, it is customary to rely on agglutination as a test of the efficiency of the antiserum. Accordingly, when our original supply of antiinfluenza serum agglutinated a certain meningeal strain of the influenza bacillus up to a dilution of 1/1000, we assumed that it would be effective as a bactericidal serum. After some months, however, we began to have some doubts, especially when Dr. Margaret Pittman tested it and found that it contained no precipitins. It was at this time that we began to investigate the whole problem more thoroughly,

TABLE II

No. of organisms in tube	Concentration of Antiserum A					Concentration of Antiserum B					Control, no antiserum
	1/25	1/250	1/2,500	1/25,000	1/250,000	1/25	1/250	1/2,500	1/25,000	1/250,000	
200,000	+	+	+	+	+	+	+	+	+	+	+
20,000	+	+	+	+	+	+	0	0	+	+	+
2,000	0	+	+	+	+	+	0	0	+	+	+
200	0	+	+	+	+	+	0	0	0	+	+
20	0	0	0	0	0	+	0	0	0	0	0

and obviously one of the first things was to test the bactericidal action of the antiserum.

In setting up the experiment, the results of which are shown in Table II, we compared the bactericidal action of the original antiserum (Antiserum A) agglutinating *B. influenzae* in a dilution of 1/1000, with serum taken some months later from the same horse (Antiserum B). The horse was being regularly injected with formalized cultures throughout all this period. Antiserum B agglutinated the same meningeal strain of *B. influenzae* up to a dilution of 1/8000.

Normal rabbit blood diluted with equal parts of 5 per cent digested blood broth was used as the source of complement. (Each tube contains 0.5 cc. of this rabbit blood broth mixture, a known amount of antiserum and a known number of organisms. The tubes were sealed, incubated, then broken open and plated out. The details of this technique have already been referred to.

Table II shows that the original Antiserum A, although it had an agglutination titre of 1/1000, had a bactericidal action in a concentration of 1/25 only (compare with control); whereas Antiserum B, with an agglutination titre of 1/8000, had a

The results of the above experiment and the proved absence of complement in the cerebrospinal fluid in this disease showed that a modification of the treatment was essential, since although leucocytes are present in the cerebrospinal fluid, nothing but a very feeble bactericidal action could be brought about by the injection of antiserum alone.   The obvious change that was indicated was the introduction of complement along with the antiserum, as indeed had been advocated by Kolmer (6).   This method of treatment has now been tried on 8 cases.

In the first 5 cases (which are reported in another paper) some improvement took place temporarily, although all of the 5 patients died eventually.   In the next 2 cases, the disease was very far advanced on admission to hospital, and not even temporary improvement was noted.   The most encouraging feature of the first 5 cases was the fact that the cerebrospinal fluid was sterilized at one time or another during the disease, in 1 case for 5 days, in a second case for 10 days and in a third case for 14 days.   Clinical improvement coincided with the temporary sterilization of the cerebrospinal fluid.   Autopsy findings in these cases showed that abscesses were definitely walled off from the general subarachnoid space, and presumably the walls of the abscess protected the organisms from the action of the antiserum and complement.   The eighth case, reported to us by Dr. A. Kuttner of the Johns Hopkins Hospital, was a child aged 2½ years, admitted 3 days after the onset of influenzal meningitis.   The child was treated daily with the influenzal antiserum and human complement.   The cerebrospinal fluid was found to be sterile 24 hours after the first treatment, it remained sterile and the child recovered.

It is clear from these cases that it is possible to sterilize the main subarachnoid space, provided the treatment is commenced reasonably early in the disease, but it is not clear how the formation of abscesses is to be prevented or how they are to be treated once they have formed, because their site at the base of the brain taxes the technique of the most highly skilled brain surgeon.   To prevent the formation of abscesses in this region, Dr. F. D. Ingraham and Dr. L. D. Fothergill are attempting to find a better method of introducing the antiserum and complement than by simple lumbar or ventricular injection, and provided the cases are diagnosed early enough—this is perhaps the greatest of the difficulties one has to contend against—they have not lost hope of eventually being able to overcome the obstacle of abscess formation.

Meanwhile, it is our part to make the mixture of antiserum and

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2,000	0	+	+	+	+	+	0	0	+	+	+
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20,000	+	+	+	+	+	+	0	0	+	+	+ +
2,000	0	+	+	+	+	+	0	0	+	+	+ +
200	0	+	+	+	0	+	0	0	0	+	+
20	0	0	0	0	0	+	0	0	0	0	0

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Table II shows that the original Antiserum A, although it had an agglutination titre of 1/1000, had a bactericidal action in a concentration of 1/25 only (compare with control); whereas Antiserum B, with an agglutination titre of 1/8000, had a

slight bactericidal action in a concentration of 1/25,000.   In other words, a serum which was but eight times stronger in its agglutinating action was approximately a thousand times stronger in its bactericidal action.   This discrepancy is analysed in Paper II.

We need hardly say that from this time onwards Antiserum B was used in the treatment of the cases.   Actually, it was used for the first time on the first of the 8 cases previously mentioned.

It will be noticed in Table II that the strong Antiserum B shows the Neisser-Wechsberg phenomenon very plainly, in that the strong concentration of this antiserum has no bactericidal action at all, in contrast to the marked action of the weak concentrations.   The reasons for this phenomenon are still in dispute, but is has been known for a long time that for optimal action, complement and antibody have to be present in certain definite proportions to one another.   These experiments are always carried out *in vitro*, and we do not know for certain whether this law holds good *in vivo* as well, but if and until there is definite evidence to the contrary, we must assume that it *is* true *in vivo*.   And here in the treatment of influenzal meningitis, when we attempt to introduce a bactericidal mixture of complement and antibody into the subarachnoid space, we come face to face with the practical application of the law.   In other words, how much antiserum and how much complement are we going to inject, so that the mixture shall have the optimal effect?   The answer to this question is not a matter of working out a simple problem in arithmetic, as may appear at first sight.   The dilution factor, the diffusion factor and the absorption factor in the cerebrospinal fluid are all uncertain.   Further, we have evidence that the antiserum forms a specific precipitation with the cerebrospinal fluid, and we would suspect that the antiserum would be thereby weakened, not to speak of the probability that complement would be adsorbed by this specific precipitate.   We have indeed noted in one case, where 12.0 cc. of fresh, human serum (active as complement in a final concentration of 1/150 *in vitro*) was injected by the lumbar route, not a trace of complement could be detected in the cerebrospinal fluid 2 hours later.   It must be admitted that so far we have not had the courage to advise a change in the proportion of antiserum to complement, and 15 cc. of antiserum and 6 to 8 cc. of fresh, human serum have been injected twice a day throughout the

disease. Most of the cases responded favourably at first, and one has to be very sure of a theory to change a treatment under these conditions. However, the above proportion is probably far from the optimal, and in the light of the ultimate fate of most of these cases, some modification in the proportion of antiserum appears to be indicated.

In order to gain as much information as possible on this point, we have recently carried out some experiments designed to simulate the conditions in the cerebrospinal fluid.

The experiments have shown that the stronger the concentration of complement, the better the sterilizing effect, so that in the experiment shown in Table III we

TABLE III

No. of organisms in tube	Fresh human serum + concentration of antiserum					Fresh guinea pig serum + concentration of antiserum				
	1/15	1/150	1/1,500	1/15,000	0	1/15	1/150	1/1,500	1/15,000	0
7,000,000	++	+++	+++	+++	+++	+	0	+++	+++	+++
700,000	+++	+	+++	+++	+++	+	0	+++	+++	+++
70,000	++	+	++	+++	+++	+	0	+++	+++	+++
7,000	++	0	+	++	+++	++	0	+	0	+++
700	++	0	0	++	+++	+	0	+	0	+++
70	+	0	0	++	+++	++	0	0	0	+++
7	+	+	0	++	+++	+	0	0	0	+++

have worked with a complement concentration of 1/7.5 in saline. Saline was used as the diluent instead of the special broth, because although the organisms do not grow out as well, and the results are more irregular, saline approximates much more closely to cerebrospinal fluid. A complement concentration of 1/7.5 was used, because that is approximately the final concentration one might expect on injecting 10.0 cc. of fresh serum into the cerebrospinal fluid of a young child. Finally, the effect of fresh guinea pig serum was compared to that of fresh human serum, because of the known high complement content of the former serum.

Each tube in this experiment then contains 0.5 cc. of a 1/7.5 dilution of fresh human serum (or fresh guinea pig serum) in saline, together with a known amount of antiserum and a known number of organisms. The tubes were sealed, incubated, broken open and plated out as before.

Despite the minor irregularities, Table III shows the Neisser-Wechsberg phenomenon again quite clearly, and indicates that with this

concentration of complement, a concentration of approximately 1/150 of this antiserum gives the optimum sterilizing effect.   On the assumption that the volume of the cerebrospinal fluid of a child is about 75.0 cc., it would suggest the injection of only 0.5 cc. of the antiserum instead of the 10.0 cc. or more that is ordinarily injected.   The experiment also shows that fresh guinea pig serum is more effective than fresh human serum.   Actually, this specimen of guinea pig serum was four times more active than the human serum, when tested on sensitized red cells.   This is consistent with our previous statement (based on other experiments) that the higher the concentration of complement, the greater the sterilizing effect.

It is fully realised that the inferences to be drawn from this experiment rest on no very secure foundation, because the experiment itself is based on the assumption that the conditions *in vitro* approximate the conditions *in vivo*, and we are very far from sure of this.   Nevertheless, one can hardly ignore the test-tube evidence that a large amount of fresh guinea pig serum with a relatively small amount of antiserum is the optimal bactericidal mixture to inject in these cases.

DISCUSSION

The results achieved so far with the specific serum treatment of influenzal meningitis compare unfavorably with the results of similar treatment in meningococcus meningitis.   Too little is known of both diseases to permit of any definite reason being given for this.   At the same time, the disease processes differ in at least two ways:

1. Complement is present in meningococcus meningitis, absent in influenzal meningitis.

2. Abscesses appear to be walled off quite early in influenzal meningitis.

Although the cerebrospinal fluid was sterilized temporarily in most of these cases, the sterilization took some days to effect, and it is probably significant that in the one case in which the sterilization was permanent, the cerebrospinal fluid was found free of organisms 24 hours after the first treatment, just as in successfully treated meningococcus meningitis.

The antiserum in the presence of complement has a strong bactericidal action *in vitro*, but the observed rapid disappearance of the in-

jected complement from the cerebrospinal fluid leaves some doubt as to the efficiency of this method of supplying the missing complement. Unfortunately, in the one case in which the fluid was sterilized immediately and the patient recovered, we have no information as to the presence or absence of complement in the fluid before treatment with antiserum and complement was commenced.

Early diagnosis in this disease is no doubt a very important factor indeed, and was perhaps the main reason why one case was treated successfully. But the cases are often in an advanced stage when admitted to hospital and the fact that even these cases sometimes show a temporary improvement under treatment gives a little hope that if the surgical and serological techniques can be improved, some of them may be cured. "Diagnosed too late" perhaps comes to the tongue too readily, if treatment is unsuccessful.

CONCLUSIONS

1. An acute purulent meningitis due to the invasion of the meninges by Pfeiffer's influenza bacillus is not a very uncommon disease in infants and young children. It has a very high mortality.

2. Complement is entirely absent in the cerebrospinal fluid of these cases, and bactericidal experiments suggest that the injection of a specific antiserum will have but slight lethal effect on the organisms unless complement is injected at the same time.

3. Treatment with a mixture of specific antiserum and complement led in some cases to a definite clinical improvement, coincident with sterilization and clearing of the cerebrospinal fluid. But after some days, the patients relapsed and died. Autopsy showed localized abscesses in the vicinity of the base of the brain, the lesions being definitely walled off from the general subarachnoid space. In one case, the patient recovered.

4. Since the walls of the abscesses apparently present an insuperable mechanical obstacle to the action of the antiserum and complement, the possibility of preventing the formation of abscesses is discussed. Earlier diagnosis and more rapid sterilization are the most obvious measures. Bactericidal experiments indicate that the proportion of antiserum to complement may be an important factor in bringing about a more rapid elimination of the bacilli.

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STUDIES ON INFLUENZAL MENINGITIS

II. B. INFLUENZAE—THE PROBLEM OF VIRULENCE AND RESISTANCE*

BY JOYCE WRIGHT, B.M., B.CH., AND HUGH K. WARD, M.B.

(From the Department of Bacteriology and Immunology of the Harvard University Medical School, Boston)

In an admirable account of the variations that occur in the bacterial species *B. influenzae*, Pittman (1) has described recently two forms. One form grows as a smooth colony, is virulent for animals, forms a soluble substance which precipitates the corresponding antiserum and the organisms tend to be uniform in size and appear to have a capsule—this is the S form. The other form grows as a rough colony, is non-virulent for animals, does not form a precipitating soluble substance, and the organisms tend to be less uniform in size and have no capsule—this is the R form. Further, two types of the S form are described, distinguished by two different soluble substances, as in the case of the pneumococcus. But as the meningeal strains fall into one type—and that has been our own experience—this type specificity, though very important in a general survey of the pathogenic members of the species, need not concern us here.

Primarily interested in the invasion of the meninges by virulent organisms and the means that can be devised to aid the body in overcoming this invasion, we have been working on the same problem from a slightly different angle. In other words, the particular problem we wished to study was virulence and resistance, and the relation of the one to the other.

Virulence

Pittman (1) pointed out that it was not easy to demonstrate the difference between virulent and non-virulent strains by inoculation into animals, because of the toxicity of both cultures, and the lack of

an animal which was really susceptible to invasion by the virulent organism. The minimum lethal dose for the mouse was quoted as 0.1 to 0.5 cc. of the S culture, and 1.0 to 2.0 cc. of the R culture. A difference of the same order was found in rabbits. This is not very convincing, and if one had to rely on such a test to distinguish a virulent from a non-virulent culture, minor differences in suceptibility in the test animals might easily lead to error. However, we have found that it is possible, by using diluted blood in a bactericidal blood test, to demonstrate a difference between the S and R cultures of the same high order of magnitude as is found in injecting mice with S and R cultures of pneumococci.

TABLE I

S culture		R culture	
No. of organisms in tube	Growth	No. of organisms in tube	Growth
3,000,000	++++	9,000,000	++++
300,000	++++	900,000	0
30,000	++++	90,000	0
3,000	++++	9,000	0
300	++++	900	0
30	++++	90	0
3	++++	9	0

Here, and in the following tables, ++++, +++, ++, + = various degrees of growth. 0 = sterility.

The technique for such an experiment has already been described in Paper I. Each tube contains 0.5 cc. of a mixture of equal parts of fresh defibrinated rabbit's blood and 5 per cent digested blood broth and a known number of organisms. The S culture was isolated from a case of influenzal meningitis and had been kept in rabbit blood in the manner described in Paper I. The R culture was grown from an R colony derived from the S culture by cultivating the S strain in 5 per cent rabbit blood peptone water for a number of transplants. Both S and R cultures were carefully checked and conformed with all the usual tests. The results of the experiment are shown in Table I.

Table I shows that diluted rabbit's blood can kill approximately one million times as many R organisms as S organisms. Other experiments have shown that if the rabbit blood is not diluted, the difference is less marked, as the undiluted blood can kill some thousands of S

organisms. This is consistent with the *in vivo* resistance to infection. And diluting the blood with suitable culture medium may be likened to lowering the resistance to infection of the whole animal. Of course it can be argued, and no doubt rightly so, that such a test is not a real virulence test, because the word "virulence" implies virulence towards a healthy animal. But if there is no healthy animal available which is susceptible to the organism, and no consistent method of lowering resistance *in vivo*, then such a technique is the closest approximation to a virulence test that is possible at present.

The next question, naturally, is why is the S organism virulent, and the R organism non-virulent. Pittman (1) has described the presence of capsules round the S organisms, and although we have noted the same appearance on occasion, and felt reasonably sure that there was a definite structural difference between the S and R organisms stained with the same stain on the same slide, on other occasions we have not felt so certain. Capsule stains are difficult, and faults in technique may well explain the inconsistency of our results. Be that as it may, from our point of view, the material difference between the two strains was of more importance than the structural difference. Here the evidence pointed to an investigation of the precipitinogen (or "soluble substance" as it has been called) which could be demonstrated in S cultures, but not in R cultures. Table I shows the resistance of S organisms to the bactericidal action of blood. If we can show that this resistance can be still further enhanced by the addition of the filtrate of an S culture, but not by the filtrate of an R culture, then presumably the active substance of the filtrate is present also in the S bacilli and is associated with their virulence. That this is the case, is shown in Table II.

In this experiment, 0.5 cc. of undiluted rabbit blood was used in each tube, instead of diluted blood, as in the last experiment, because unless some bactericidal action was shown by the blood alone, the effect of adding the S filtrate could not be demonstrated. The filtrates were prepared by passing 18 hour cultures in the special broth through Berkefeld filters.

The experiment of which the results are shown in Table II establishes the fact that there is in the S filtrate a substance which has an antibactericidal effect. The same substance is almost certainly in

the S bacilli themselves, and although no direct proof can be given, this substance is probably intimately associated with the virulence of the organisms.   The next question, of course, is: What is the active substance in the S filtrate which has this effect?   We have already tentatively assumed that it is the precipitinogen, but as yet this has not been proved.   On the analogy of other organisms, such as the pneumococcus and Friedländer bacillus, one would suspect that the precipitinogen of the S influenza bacillus would be found to be a carbohydrate.   Goebel, indeed, is quoted by Pittman (1) as having already demonstrated that it is a carbohydrate.   Our next experiment was designed to test the theory that the antibactericidal substance in the S filtrate is a carbohydrate.   If it is a carbohydrate, then it should be

TABLE II

No. of S organisms in each tube	Concentration of S filtrate			Concentration of R filtrate			Control (blood only)
	1/15	1/60	1/240	1/15	1/60	1/240	
15,000,000	++++	++++	++++	++++	++++	++++	++++
1,500,000	++++	++++	++++	++++	++++	++++	++++
150,000	++++	++++	++++	+	0	0	0
15,000	++++	++++	0	+	0	0	0
1,500	++++	++++	0	0	0	0	0
150	++++	++++	0	0	0	0	0
15	++++	++++	0	0	0	0	0

able to withstand heating.   Accordingly, we boiled the same filtrate that had been used in the previous experiment at pH 9.4, neutralized, and repeated the experiment, using the heated S filtrate in place of the unheated filtrate.   The results of this experiment showed that the heated filtrate had practically no antibactericidal effect.   At the same time, corroboration was given to the theory that the precipitinogen is a carbohydrate by the fact that the precipitinogen content of the filtrate was unchanged by heating.   On the face of it, these two facts seem to indicate that the substance in the filtrate which has the antibactericidal effect is not the precipitinogen, but another heat-labile, specific substance.   There is, however, another possibility.   It may be that the precipitinogen in the unheated filtrate is not a pure carbohydrate, but a more complex substance which is much more active

than the carbohydrate in its antibactericidal effect. On heating, this hypothetical compound is split up, leaving the carbohydrate. On this theory, the complex substance and the pure carbohydrate would have the same precipitinogen content, but the pure carbohydrate would have a weaker antibactericidal effect. This theory sounds somewhat far-fetched, but one of the authors has more definite evidence in the case of the pneumococcus (to be published shortly), which points in this direction. When we have available a supply of the purified influenza bacillus carbohydrate, we will be able to determine whether the carbohydrate itself in stronger concentration has an antibacteri-cidal effect. In an 18 hours heated filtrate, its concentration—as determined by the precipitin test—is very weak, and if its action is similar to that of the pneumococcus carbohydrate, one would not expect it to have an antibactericidal effect in that concentration. And there for the moment we have to leave the question of viru-lence. Almost a *sine qua non* for further work is a supply of the purified carbohydrate.

Resistance

In any attempt to develop a bactericidal method of treating in-fluenzal meningitis, a reliable technique for testing the bactericidal efficiency of the antiserum must sooner or later be found. In our case, unfortunately, it was later. In the beginning we relied upon the agglutination test, and it was only when we began to test the bac-tericidal action of antiserum, that we realised how misleading the agglutination test may be. Table I of Paper I shows the result of testing two antisera. Antiserum A agglutinated the organisms up to a serum dilution of 1/1000, but was only active in killing the organ-isms in a concentration of 1/25, whereas Antiserum B agglutinated up to a dilution of 1/8000 but had a slight bactericidal action in a concen-tration of 1/25,000. Thus an antiserum which was only eight times stronger in its agglutinating action was approximately a thousand times stronger in its bactericidal action. As was pointed out to us originally by Dr. Pittman, Antiserum A contains no precipitins at all against the precipitinogen of the S influenza bacillus, whereas Anti-serum B formed a precipitate with the precipitinogen up to a dilution of 1/64. This of course made us suspect that the bactericidal action

of the antiserum was much more intimately associated with the precipitin antibody than it was with the agglutinin antibody. Such a theory is consistent with recent studies by Friedlander, Sobotka and Banzhaf (2), and by Heidelberger, Sia and Kendall (3), working with antipneumococcus serum.

This quantitative discrepancy between the agglutinins on the one hand and the precipitins and bactericidins on the other is suggestive, but does not constitute proof that the agglutinating antibody is quite distinct from the antibody responsible for precipitation of the precipitinogen and the killing of the organisms in the presence of complement. It occurred to us that if we could by some means (1) absorb the agglutinins only from an anti-S influenza bacillus serum, leaving the precipitins and bactericidins unchanged, and (2) absorb the precipitins and bactericidins only from an anti-S influenza bacillus serum, leaving the agglutinins unchanged, such a demonstration would be strong evidence that the agglutinin was a separate and distinct antibody from the precipitin-bactericidin antibody, and not the same antibody acting under different conditions. Even if only one of these demonstrations was possible, it should suffice, because the one is the corollary of the other. Actually, the technique for the second demonstration is probably easier than for the first, but a supply of purified precipitinogen (presumably a carbohydrate) would be theoretically necessary to absorb the precipitins and bactericidins without affecting the agglutinins, and this we did not have, nor know how to obtain. We tried, but eventually convinced even ourselves that we had neither knowledge nor skill in chemistry. Accordingly, we had to attempt the first demonstration. For this, if we were to absorb the agglutinins from an antiserum and leave the precipitins and bactericidins untouched, it is clear that we had to have an absorbing antigen which was strong in agglutinogen and completely lacking in precipitinogen. Fortunately we had such an antigen ready to hand in the R influenza bacillus previously mentioned in this paper. We found that the R strain was agglutinated just as well as the S strain by the anti-S serum, and we could find no trace of precipitinogen in the R organism. All that was essential in carrying out the experiment was to absorb the anti-S serum with the R organism and compare the R-absorbed serum with the unabsorbed serum by means of quantitative agglutina-

tion, precipitation and bactericidal tests. But at the same time we thought that if we included in the comparison the anti-S serum absorbed with the S organism, it would be an additional check on the technique. Theoretically the S-absorbed anti-S serum should show a definite weakening in agglutinins, precipitins and bactericidins, because the S organism contains both agglutinogen and precipitinogen.

The actual technique of this experiment was as follows: The anti-S serum used was fairly strong, both in agglutinins and precipitins. The organisms for adsorption were obtained by growing each strain on chocolate agar in Kolle flasks, washing off the growth with saline and centrifuging down the organisms. The bacilli from one Kolle flask were used for absorbing each 1.0 cc. of antiserum. The antiserum was added to the centrifuged bacilli, mixed and left in the ice box for 48 hours. This process was repeated four times. At the end of this time, we thus had three specimens of anti-S serum for testing: (1) an S-absorbed anti-S serum, (2) an R-absorbed anti-S serum, (3) an unabsorbed anti-S serum. The agglutination test was carried out by adding an equal volume of an 8 hour culture of the S strain in 5 per cent digested blood broth to the various dilutions of the anti-serum, and incubating in a water bath at 50°C. for 18 hours before reading.[1] The precipitation test was carried out by adding an equal volume of the Berkefeld filtrate of an 18 hour culture of the S strain in 5 per cent digest blood broth to the various dilutions of the antiserum, and incubating for 2 hours at 37°C. before reading. The bactericidal test was performed as previously described. Each tube contained 0.5 cc. of a mixture of equal parts of fresh rabbit blood and 5 per cent digest blood broth, a known amount of antiserum and a known amount of organisms. The tubes were sealed, incubated, broken open and plated out. The results of testing these three specimens of antiserum for agglutinins, precipitins and bactericidins are shown in Tables III and IV.

It will be seen from Tables III and IV that the effect of absorbing an anti-S influenza bacillus serum with the R strain of the influenza bacillus is to leave the precipitins and bactericidins unchanged and at

[1] In carrying out the agglutination test in this manner, two different kinds of agglutination are seen. In the stronger concentrations of the antiserum, the agglutination is floccular in character, but as the serum becomes more dilute, the agglutination is in the form of fine but perfectly definite granules. The floccula-tion type of agglutination ends at about the same serum dilution as the precipita-tion reaction ends with the broth filtrate. Whether the organisms themselves agglutinate in this way owing to their content of precipitinogen, or whether the precipitate in the suspending medium (the suspending broth contains the pre-cipitinogen and therefore precipitates with the antiserum) entangles the organisms and carries them down has yet to be determined.

the same time to remove most of the agglutinins. The agglutination that was still present may have been genuine agglutination or it may have been primarily a precipitation (see footnote 1). But accepting

TABLE III

Concentration of antiserum	Agglutination test			Precipitation test		
	Unabsorbed antiserum	S-absorbed antiserum	R-absorbed antiserum	Unabsorbed antiserum	S-absorbed antiserum	R-absorbed antiserum
1/4	++++	+	++++	+++	0	++±
1/8	++++	±	+	++±	0	++
1/16	++++	0	+	++	0	+±
1/32	++++	0	+	+	0	+
1/64	++++	0	+	±	0	±
1/128	++++	0	+	0	0	0
1/256	++++	0	±			
1/512	++++	0	0			
1/1,000	++++	0	0			
1/2,000	+++	0	0			
1/4,000	++	0	0			
1/8,000	±	0	0			
1/16,000	0	0	0			
Control	0	0	0	0	0	0

TABLE IV

Bactericidal Test

No. of organisms in each tube	Concentration of unabsorbed antiserum				Concentration of S-absorbed antiserum				Concentration of R-absorbed antiserum			
	1/15	1/150	1/1,500	1/15,000	1/15	1/150	1/1,500	1/15,000	1/15	1/150	1/1,500	1/15,000
300,000	++++	+++	++	++++	+++	++++	++++	++++	+++	++	+++	++++
30,000	++++	0	0	++++	+++	++++	++++	++++	+++	0	0	++++
3,000	++++	0	0	++++	+++	++++	++++	++++	+++	0	0	++++
300	++++	0	0	++++	+++	++++	++++	++++	+++	0	0	++++
30	++++	0	0	++++	+++	++++	++++	++++	+++	0	0	++++
3	++++	0	0	++++	+++	++++	++++	++++	+++	0	0	++++

it as genuine agglutination, only 3 per cent of the agglutinins remained. The effect of absorbing the same antiserum with the S organisms was to remove agglutinins, precipitins and bactericidins.

These results appear to us to constitute strong evidence that the

agglutinogen is a separate antigen, and the agglutinin a separate antibody. As we have stated already, the corollary of this experiment was beyond our reach, since we had no purified precipitinogen with which to absorb the precipitins. However, we have carried out an obvious experiment which, though hardly as convincing as absorbing the precipitins out of an anti-S serum, must carry some weight.

Two rabbits were immunized, one with the S strain and the other with the R strain of the influenza bacillus. The anti-S serum and the anti-R serum thus obtained were compared in the same manner as before—by their agglutination of the S bacilli, by their precipitation of the S filtrate and by their bactericidal action on the S organisms. The protocols of this experiment need not be given, as the technique was the same, and the results were quite clear-cut. The anti-R serum agglutinated the S bacilli equally as well as the anti-S serum did—the end-point being a serum dilution of 1/8000 in each case—but the anti-R serum showed no precipitating or bactericidal action, whereas the anti-S serum was strong in precipitins and bactericidins.

If both these experiments indicate that the agglutinogen of the influenza bacillus is a separate antigen, and the agglutinin a separate antibody, they establish the identity of precipitin and bactericidin more firmly than ever, if, indeed, that were needed. This is not the place to venture into a wider field than that of the influenza bacillus, but the experiments suggest that the presence of precipitins is of more significance than the presence of agglutinins in testing bactericidal sera, provided always that one uses the right precipitinogen. The importance of the latter point is perhaps not always fully appreciated.

DISCUSSION

In the case of an organism like the diphtheria bacillus, the close relationship between virulence and resistance is quite evident. The organism is virulent mainly by virtue of the toxin it produces, the individual is resistant mainly by virtue of the antitoxin present in the serum. Toxin-antitoxin; virulence-resistance. But in the case of the influenza bacillus, the connection between virulence and resistance is not so easily demonstrated. Were we to assert that the virulence of the S influenza bacillus is dependent on the presence of precipitinogen in the organism, and resistance dependent on the presence of precipitin in the serum, we would have to admit at the

same time that we have no definite proof of this very elementary hypothesis, which may be stated just as simply as in the case of diphtheria, *viz.*, precipitinogen-precipitin; virulence-resistance. As the problem stands at present, however, the experimental data lend considerable support to this theory.

With regard to virulence, it has been shown that the virulent organism is completely resistant to the bactericidal section of dilute normal blood, while the non-virulent organism is very easily killed by dilute normal blood; we know that the filtrate of the virulent organism contains the precipitinogen, while the filtrate of the non-virulent organism does not contain the precipitinogen; it has been shown that the filtrate of the virulent organism is strongly antibactericidal, while the filtrate of the non-virulent organism has no antibactericidal effect. These facts suggest the identity of the precipitinogen and the antibactericidal substance. Against this hypothesis must be set the fact that the precipitinogen is stable, while the antibactericidal substance is unstable on heating at pH 9.4. It is possible, however, that the unheated precipitinogen is a more complex form of the precipitinogen proper (presumably a carbohydrate) and in this complex form is much more active in its antibactericidal effect. This point can only be settled when the purified precipitinogen is available.

With regard to resistance, it has been shown that the antiserum has a bactericidal action in the presence of complement only when it contains the precipitin antibody, and we know that the precipitin antibody combines with the precipitinogen in the form of a precipitate, and presumably neutralizes it. We can then picture the virulent organism as containing the precipitinogen (probably concentrated in the capsule or membrane) and we can picture this substance protecting the bacillus from the action of the complement. If, however, the precipitin is present, this antibody neutralizes the protecting covering of precipitinogen and lays the bacillus open to the action of the complement. Pursuing this subject still further, we should of course want to know why this covering should protect the virulent bacillus from the action of the complement. Mudd, Lucké, McCutcheon and Strumia (4), working on the mechanism of phagocytosis, have lately thrown considerable light on a similar problem. It is hoped that direct studies of this nature, and the more indirect in-

vestigations of the action of the purified soluble substance (when it is available) will help to clarify the problem of virulence and resistance in the case of the influenza bacillus.

In studying and discussing the question of resistance, we have laid all the emphasis on the action of the bactericidal antibody and complement, and we believe it is the critical mechanism of resistance, but at the same time we are not blind to other processes which aid the body in eliminating the invading organisms. For example, we have rather discounted the effect of agglutinins in resistance, but it was their relative unimportance rather than their absolute unimportance that we intended to stress. It may well be that the agglutinins have an important function *in vivo*, that we cannot demonstrate in test-tube experiments.

CONCLUSIONS

1. By a suitable bactericidal technique, it can be demonstrated that the virulent S influenza bacillus is completely resistant to the bactericidal action of diluted normal unheated serum. In contrast, the R organism is easily killed when subjected to the action of diluted normal serum. Although this is not a true virulence test, it promises to be a useful substitute when a susceptible animal is not available.

2. The S culture filtrate contains a substance with a strong antibactericidal effect, but the R culture filtrate does not contain this substance.

3. It would appear probable that this antibactericidal substance is identical with, or closely related to the precipitinogen (or soluble substance) which is present only in the culture fluid of the S influenza bacillus. In view, however, of differences in heat stability of the two substances, this question must remain in doubt until a supply of purified precipitinogen is available.

4. Quantitative agglutination, precipitation and bactericidal comparisons between (*a*) R-absorbed anti-S serum and unabsorbed anti-S serum and (*b*) anti-S serum and anti-R serum indicate that the agglutinogen is a separate antigen, and the agglutinin a separate antibody, taking no part in the bactericidal action of the antiserum. They also indicate that the precipitin, which is present only in the anti-S serum, is identical with the bactericidal antibody.

5. The relation of the precipitinogen (or soluble substance) to virulence and of the precipitin to resistance is discussed.

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THE INCIDENCE OF CANCER IN TARRED AND SHELTERED MICE

By PEYTON ROUS, M.D., AND ELIZABETH BOTSFORD

(From the Laboratories of The Rockefeller Institute for Medical Research)

The experiments to be described here prove that tar cancer develops with the same frequency in mice sheltered throughout a long period of adult life from the access of living entities by way of food, drink, air, or bedding as in exposed controls. They would appear to rule out the possibility that the disease is due to an infectious agent reaching the body from its surroundings during the period when cancer ordinarily develops. Such being the case, one can discuss with the less reserve the assumption necessary to the experiments, that cancer is of infectious origin.

Theoretical Considerations

No explanation of the cause of cancer is worthy of attention that cannot be tested. A main attraction of the hypothesis that the disease is an infection lies in its accessibility to test. Yet clearly the supposititious infectious agent can be no ordinary one. The conclusive evidence, both statistical and experimental, against contagion in cancer, the manifest growth of neoplasms *aus sich heraus*,—to use Ribbert's phrase,—and the failure of the numerous efforts to demonstrate causative agents in mammalian tumors have made plain the fact that a living entity responsible for such growths must require for effectiveness a very special basis of predisposition. Local changes preliminary to cancer have long been recognized. Tissue derangement has been shown to precede malignancy in most cases, with the likelihood that it does in all. The proof by the geneticists that certain stocks or families are prone to have cancer may mean no more than that there exists a heritable tendency to local tissue derangement of the sort which "leads to cancer," the manner in which it leads being still unknown. Indeed a recent study of the differences in involution of the mammary gland of cancerous and non-cancerous strains of mice

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has yielded an illustrative instance of the sort (1). If it were possible to raise under aseptic conditions a strain of animals greatly liable to malignant disease, and to show that under such conditions these animals never have it, one would still be unable to conclude that the disease is infectious in nature, since the conditions of the aseptic life might have ruled out, not a cancer parasite, but merely certain causes of the tissue derangement leading to malignant growth (as *e.g.*, the nematode worms which cause the injury upon which sarcoma develops in the liver of rats). In every attempt to test the hypothesis that cancer is due to infection, tissue deranged in such a way that the etiological agent can act upon it must be provided. The process of tarring the skin yields such tissue, and to tarring we have resorted in the present work.

The facts force one to suppose certain things of any hypothetical infectious agent causing neoplasms; and these must be taken into account in the planning of experiments to disclose its presence. The agent must be present frequently in the animal body and well-nigh ubiquitous in nature else tarred mice in all parts of the world could not regularly become cancerous. On the other hand, it can only occasionally reach cells susceptible to its action, or the cells must rarely become susceptible, since otherwise neoplastic change would occur broadcast in tarred tissue, instead of in foci which are often solitary, generally punctate, and which give every evidence of having arisen by proliferation from a single cell or a small group of cells. Beyond causing these elements to proliferate and to become abnormal in morphology and behavior, the extraneous causative agent must play but a subordinate rôle in the phenomena of the disease, since these are plainly referable to the cells as such. This state of affairs has been proven to exist in the case of the chicken sarcomata, which, furthermore, are of such highly various and often elaborate histology that it would be difficult to suppose them due to causes separable from the cells were the fact not attested.

Granting the foregoing, one may assume that the hypothetical cancer-producing agent leads an existence, in or upon the host, and is harmless save upon special occasion,—like the staphylococci and the colon bacillus,—or that it passes in and out of the body frequently without causing harm save when highly unusual conditions provide

it with a foothold,—as happens with actinomyces. Great difficulties lie in the way of testing the first of these hypotheses, but not so with the second, and with this we have dealt.

General Method

In our experiments the tissue injury preliminary to cancer has been produced by tarring paired groups of young adult mice, and the animals of one group have been placed under conditions which would facilititate the entrance into the body of extraneous living agents, whereas those of the other have been sedulously protected.

The many weeks required to produce in tarred mice the changes preliminary to cancer have had their advantage in relation to the work as tending to render the differing environmental conditions effective; but on the other hand the longer the duration of the experiment the greater have been the chances that a supposititious cancer-producing organism would reach the sheltered animals. We have used tar of the sort employed by Deelman[1] and can confirm his observation that it causes cancer to develop with unusual rapidity. The animals were shaved over the entire back from neck to tail, to begin with, and were tarred over the whole area three times in each week. Between whiles the bottles of tar for the control and experimental series,—filled at one time from the same well-mixed supply,—were kept in a closed cupboard under identical conditions. The tar was applied with a spatula,—which had been sterilized in the case of the sheltered animals.

Criteria of Malignancy.—In a preliminary year the characters of tar warts and cancers were studied. These need no description. In common with previous authors we found difficulty in determining precisely when cancer supervenes upon non-malignancy. The histological appearance of tar tumors is notoriously deceptive, and so too at times is the clinical aspect. The transplantation of tar warts has shown that some of these are potentially malignant. On the other hand, growths which are ulcerated, spreading, and to all clinical appearance highly malignant, may dwindle and disappear for good if the animal sickens and loses weight rapidly as result of an intercurrent illness, or if tarring is stopped. It was decided to accept as cancers only those growths which at autopsy showed plain evidence in the gross of extension into the underlying tissues when the skin was everted, a finding confirmed by the histological features of malignancy. This basis for decision proved satisfactory. On stripping back the skin which bears ordinary tar warts one sees beneath these no induration, or extension downwards,

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[1] Horizontal retort tar of the Oster-Gasfabrik of Amsterdam (Deelman, H. T., *Z. Krebsforsch.*, 1922, 18, 261). We owe our supply to the generosity of Dr. Landsteiner. The heat used in the manufacture of this tar as well as its character effectually preclude the presence in it of a living entity productive of cancer.

but at most, and only when the wart is large, a local thinning of the subcutis such that the smooth, well demarcated, slightly puckered, shallow base of the lesion is visible. Beneath definite tar cancers, in contrast, there are translucent, grayish pink thickenings or lumps of new tissue, and these, unlike the warts just mentioned, have the characteristic histology of malignant growth. Border line cases have been rare in our experience, and in assembling the data they have been put down as non-cancerous. Often the skin over the extending malignant growths was fixed and infiltrated, and there was deep ulceration. Not a few of the cancers were recognizable as such practically from the first, having the form of discoid, subcutaneous buttons covered by adherent skin, with a minute wart near the center or an indurated erosion. The warts and cancers were charted weekly. Lung disease was frequent in our animals and lung tumors not rare. Since the interpretation of these, whether primary or metastatic, presented great difficulty, their incidence was not analyzed; nor was a census made of metastases in general. At the conclusion of each experiment the surviving animals were killed with chloroform and the adherent tar was removed from the skin with ether before it was stripped back.

Experimental Procedure.—White mice of a special strain raised in the Institute, of the same general age and condition, were placed in individual jars and separated on a weight[2] basis into two practically identical groups. Those of one group were fed ordinary diet (bread and milk and a mixture of buckwheat, dog biscuit, and rolled oats); they were kept in a dusty room in which other animals of various species lived; and were bedded upon shavings that were seldom changed, in jars seldom cleaned. The jars of the other group were placed within a closed chemical hood sealed with rubber against air currents from the quiet, clean room round about. The hood was opened only to change the jars and bedding and to feed the animals. The jars, sterilized with lysol and dried with sterile towels by a gloved operator, were changed every other day (every 3rd day when Sunday intervened), and the bedding—autoclaved shavings of the sort used with the control group,—was then renewed. At this time the animals of both groups were tarred, those of the experimental series first. When cancers had begun to appear amongst the latter, the individuals which showed it were handled last by the operator who, for this series, used sterile gloves.

The tarring of large skin surfaces is ill borne by mice. It seemed certain that the additional strain of a sterile, synthetic diet would not be tolerated. For this reason recourse was had to mammalian muscle as food for the sheltered group. The occurrence of cancer in organs which can be reached from without only by way of the blood stream indicates that a cancer-producing agent, if such there be, must now and again reach the blood and circulate therein; yet all the evidence of bacteriology attests the existence of an apparatus which removes circulating invaders rapidly and competently, with result that the blood is ordinarily sterile

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[2] All weighings were done prior to feeding.

on culture and innocuous on transfer. There was a little residual blood in the fed muscle, but exceedingly little, since this tissue in individuals bled to death, as were those furnishing the material used, becomes almost exsanguinated (2). The fresh rabbit (or beef) muscle was hacked into chunks of appropriate size, seethed in boiling water to dispose of any surface infection, and distributed with sterile tongs to the sheltered mice. Sterilized water was given the latter, in autoclaved bottles supplied with a glass drinking tube. The bottles and water were changed three times a week. Much of the fluid required by the control animals came to them in their food, but tap water, in bottles that were seldom changed or washed, was provided in addition. Their bedding was changed only every 2 weeks. Then a layer of shavings about an inch deep was placed in the jar, and more were added from time to time to avoid dampness. Contamination from house flies was excluded in the case of the experimental animals, and bed bugs and blood-sucking mites were never encountered in association with them, whereas these were not infrequent in the litter of the controls or upon their bodies.

EXPERIMENTAL

The course of spontaneous mammary cancer is in many instances much influenced by the nutritive state (3). So too is that of tar cancer, and the incidence of the disease as well (4). In our first experiment,—which yielded striking differences between the two groups of animals,—the influence of this factor and of certain other potential ones was insufficiently controlled.

Experiment 1.—It was found by test that young adult mice remain in good health when on a diet of rabbit muscle and water. Then 75 such animals were distributed in jars and separated into two groups. The individual weights ranged from 18 to 31 gm., averaging 23.4 gm. in the control animals and 23.7 gm. in the experimental group of 40 mice. At first the latter were kept upon wire daises and no bedding was provided. They were fed pieces of rabbit muscle as above described, which had been immersed in water at 75.0–80.0°C. for 3 minutes,

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Legend for Charts 1 to 7

Each line indicates the weight of a mouse and the time at which warts and cancers appeared. Only the data of those animals are charted which survived more than 60 days of tarring, that is to say to the time when warts first were seen. During the periods covered by the broken lines neither warts nor cancers appeared. A hatched line means that the mouse had become warty, and a heavy black one that cancer was present. In most instances the number designating each animal is placed at the end of the line, the point at which this is broken off indicating when death occurred; but to avoid crowding, the number is sometimes placed vertically above or below, together with a cross.
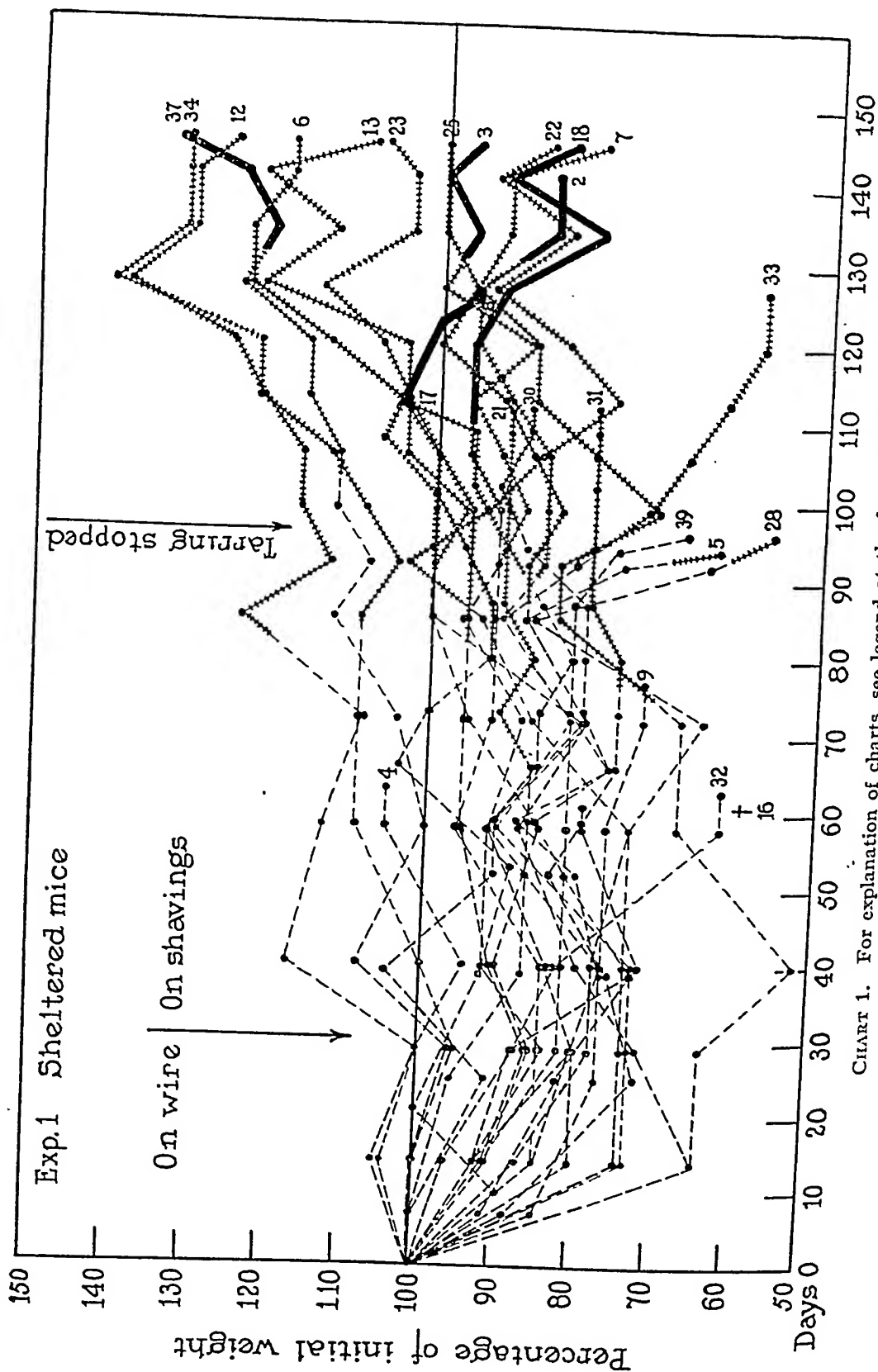
CHART 1. For explanation of charts, see legend at the foot of page 251

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CHART 2

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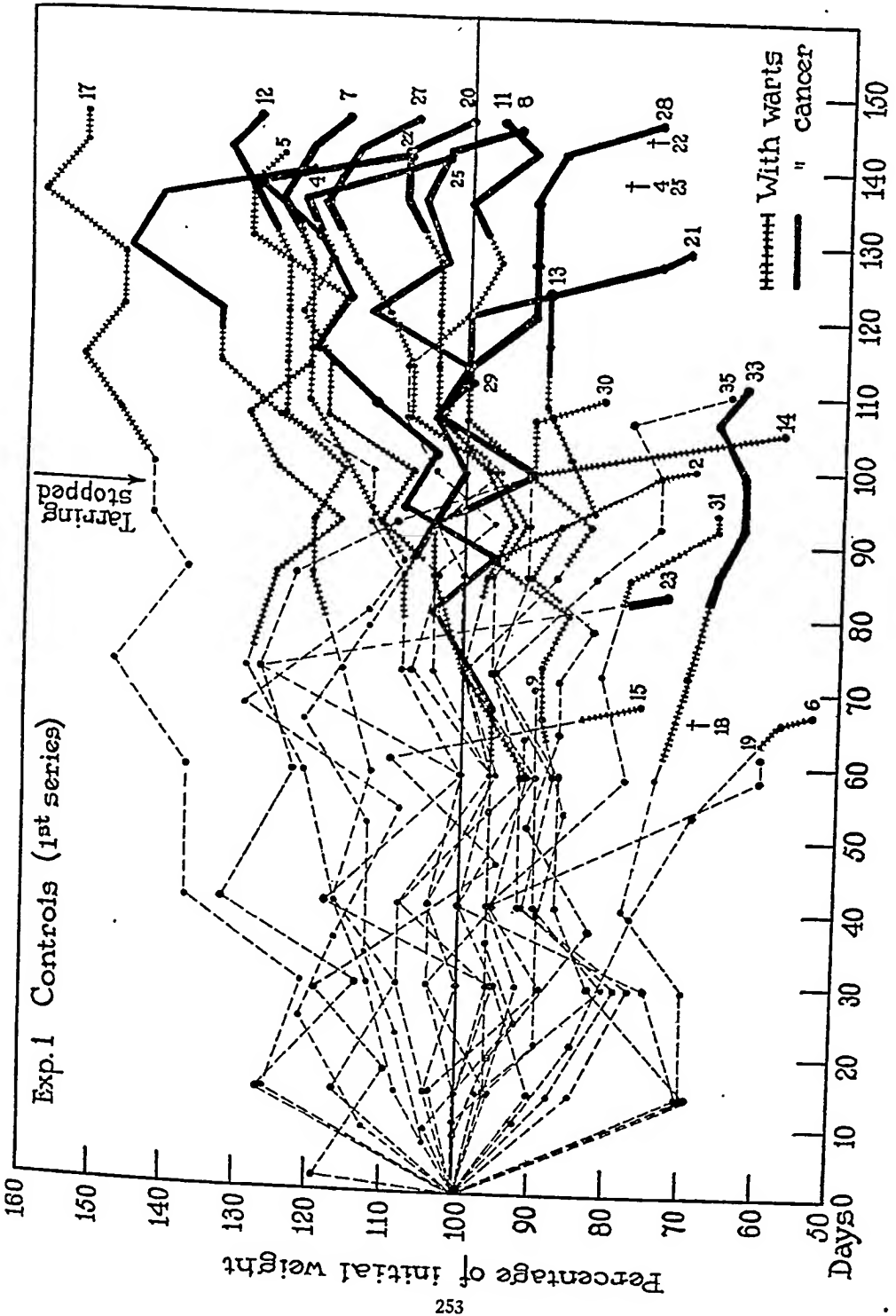
in such quantity (12 to 15 gm.) that little remained unconsumed. Saturdays they received double rations, none being given on Sunday. The meat was well taken and the small, remaining fragments dried without putrefaction. They were removed with sterile tongs at the next feeding. Some extra animals of Institute stock were kept together in a box and on ordinary diet for the replacements necessitated by deaths during the first days of adjustment to the new conditions.

On November 26, 1928, the animals were shaved and distributed in jars; next day they were weighed and grouped on the basis of the weight; and on the day following they were tarred. Weighing was done at intervals of 11 to 16 days during the first weeks, but every 7 days later on. It soon became evident that the group on the daises were doing badly. They had no place to deposit the tar they removed and hence ate much of it, while furthermore their fur tended to mat. They rapidly lost weight, the average being only 19.8 gm. after 30 days (Chart 1). The daises were then replaced with sterile shavings. A rapid improvement followed, yet on February 8 the average weight was only 20.8 gm. and on February 21 it was 22.7 gm. The controls had merely held their weight all this while, because of the tarring as later events showed; and until after March 1 the initial average was not surpassed, the controls weighing 24.0 gm. then, and the experimental series 22.1 gm. Tarring was now stopped because warts had developed in many controls and cancers in 4 of them; but the general conditions of life in both groups were not otherwise changed. No cancer had appeared in any of the experimental group at this time. The controls forthwith began to gain rapidly, reaching a maximum weight of 29.4 gm. on April 12, with a decrease later to 28.7 gm. on April 19 owing to the constitutional effects of the tumors. On April 4 the maximum average weight of 25.2 gm. for the experimental animals was reached, with 25 gm. on April 18. Both groups were killed and autopsied on April 22.

Other workers have observed that the tarred animals which develop warts soonest are those which most frequently develop cancers, the warts serving usually, though not always, as the sites of malignant change. After 67 days of tarring the first wart was noted upon one of the 22 surviving animals of the sheltered series (Chart 1). By this time already 6 of the 25 surviving controls had one or two warts, the earliest showing itself after 58 days (Chart 2). Warts continued to appear in the sheltered animals, but sparsely and slowly, whereas soon most of the controls showed them and they were often large, fleshy and numerous. The charts give no inkling of the differences in number and magnitude of the growths. The earliest cancer clinically recognizable in the 18 surviving animals of the sheltered series was noted after 110 days, at which time 7 of the 17 surviving controls already showed well advanced cancers, one having appeared after only 67 days of tarring, in the form of a rapidly growing, slightly raised, indurated button, mostly subcutaneous, with central ulceration. When the experiment was stopped after 145 days it was found that 15 of the 25 control mice surviving

when cancer first appeared amongst them had developed malignant tumors, frequently large and multiple. Two of the 10 that were free from them can be practically excluded from consideration since their backs were almost entirely covered with infected ulcers. Four of the remaining 8 bore warts, the exception being an animal which died after 70 days. Among the 22 sheltered animals surviving until after cancer had been first recognized in the controls, that is to say for more than 67 days, only 5 had become cancerous and the cancers were usually solitary. However, many had developed large warts, benign to all appearance.

In this first test both warts and cancers were far less frequent in the sheltered group than in the controls (Charts 1 and 2). In the former the growths appeared late and grew relatively slowly. But other influences besides the protected life will explain the differences. The sheltered mice were thin and in bad physical state during the first months of the tarring, some being emaciated. All were still growing, and hence the loss in weight recorded in the chart gives but a poor indication of their actual state. As already mentioned, tar warts and cancer are slow to develop in ill-nourished animals. The fact was noted in both groups that the individuals with skin most affected by the tarrings, as shown by failure of the hair to reappear during the short intervals between them, were most prone to warts and to cancer. Judged by this criterion the tarring had affected the skin of the controls much the more profoundly. After it was discontinued, their hair came in slowly and sparsely, whereas in the sheltered mice it was soon relatively abundant. In seeking a reason for the difference, we were led to check the temperature and humidity conditions more carefully, since warmth and moisture are known to enhance the carcinogenetic influence of tar. During the day these conditions were practically identical for the two series, but in the night the temperature of the room in which the controls were kept, together with rats, chickens, guinea pigs, and rabbits, sometimes rose several degrees, and the floor was not infrequently sluiced with water with result that the room became humid. Furthermore the residual food accumulating from day to day in the jars of the controls contributed to increase the humidity, a fact not infrequently attested by mold upon it.
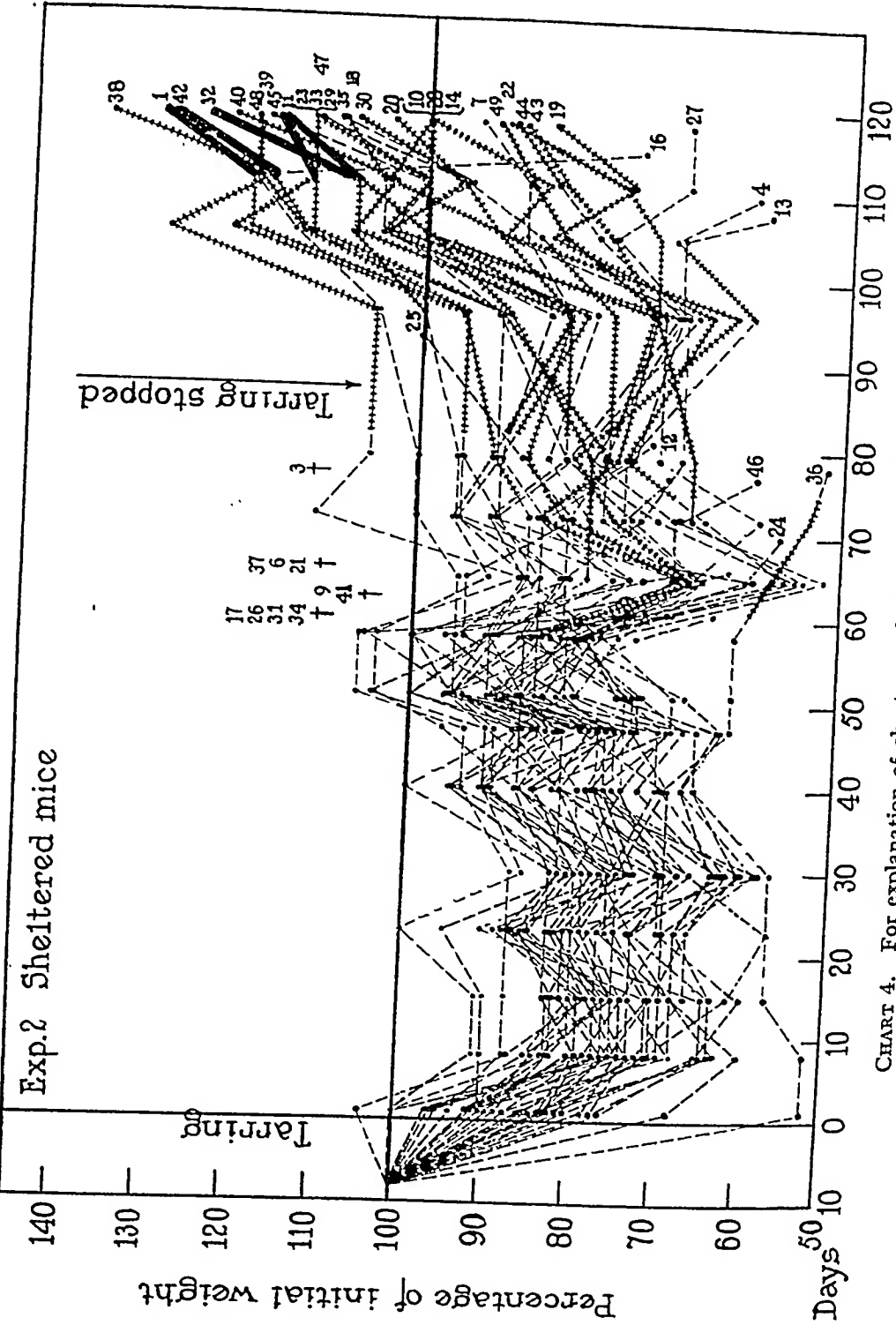
CHART 4. For explanation of charts, see legend at the foot of page 251

CHART 5
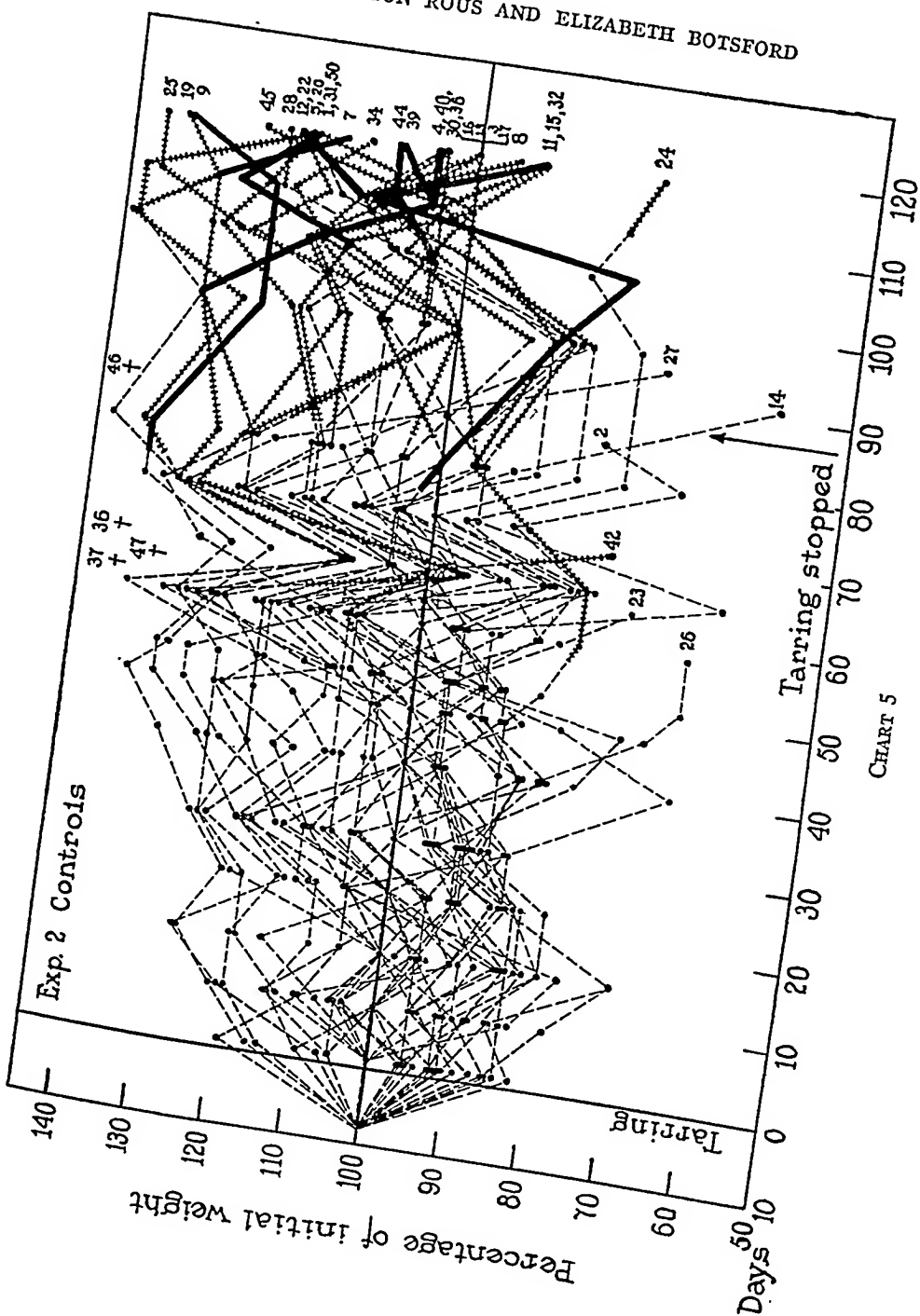
for the controls.   On April 1 and 2 the mice were shaved from nape to tail and on April 3 all were tarred.   Weighing on April 4 disclosed the fact that the mice on rabbit meat had already lost much weight, the average being only 18.7 gm. as compared with 20.3 gm. for the controls.   In the attempt to bring these latter to the weight of the sheltered animals their ration was restricted from April 8 on; but it was soon found that the procedure could not be pushed far enough to reduce the average weight to that of the sheltered animals else many would die.   It will be seen from Charts 4 and 5 that throughout the tarring period of 87 days the sheltered animals were much the thinner.   In the 39 days thereafter they rapidly made up the difference, although still sheltered; and when the groups were sacrificed after 126 days in all, the average weights were the same, 23.5 gm.   This had been brought about only by continued restriction of the diet of the controls.

The sheltered mice were kept as previously in a closed but spacious hood situated in a large quiet room, while the controls were placed in a well-ventilated room of the same suite, one in which rabbits were kept and into which other animals were frequently brought.   It was of purpose seldom cleaned.   The day and night temperatures were practically identical in the two places.

To do away with the difference in amount of tar in the jars of the two groups, those supplied to the sheltered mice were sprayed with tar at each changing, and the sterile shavings as well.

The first warts were noted in 2 controls, after 56 days of tarring; and 2 days later several were found upon one of the sheltered animals.   By the time tarring was stopped (after 87 days) 17 animals out of 31 surviving in the sheltered group had developed warts, as compared with 18 of the 31 controls.   However, in the latter, which were the better nourished, the warts were much more frequently multiple and, growing faster, were soon far larger.   In 2 of the group that were notably plump large ulcerating carcinomas had appeared in 75 days.   The first cancers were recognizable in the sheltered animals 36 days later, that is to say 24 days after tarring had been discontinued.

When the animals were sacrificed, after 118 days, 23 of the 27 surviving sheltered mice had warts and 5 of these showed cancers as well.   Of the 29 surviving controls 27 had warts and 8 cancers.   The warts were far larger than in the sheltered mice and the cancers had grown far more rapidly and destructively, being often multiple furthermore.

After tarring was discontinued, hair reappeared sooner in the experimental group than in the controls, and at the end a larger proportion had backs covered with it.

In this experiment (Charts 4 and 5) it proved impossible to keep both series of mice in the same nutritive state, but other manifest differences complicating the previous experiment were excluded. The failure to maintain comparable weights was due, as later results showed, to the youth of the animals.   About the same proportion of

the thin, sheltered mice and the relatively plump controls developed warts during the period of the tarring; but this proportion fails to illustrate the actual differences observed. In the animals of the sheltered group the warts were few, small, and indolent; whereas in the controls they appeared in relative abundance, enlarged rapidly and some soon became cancerous, the cancers being frequently multiple, notably in the plump individuals. Since our criteria of malignancy were mainly gross ones it was inevitable under the circumstances that the animals providing the more favorable conditions for the development of recognizable cancers should appear to be the more liable to the disease. The disease was recognizable in 5 out of 31 of the sheltered mice that survived the tarring, and in 8 of 31 controls.

The same tar was used as in the previous test, and mice of the same stock and age; but the tarring was kept up for only 87 days, instead of 96, and the animals were killed after 118 days instead of 145. That these differences were responsible for the smaller incidence of cancer is plain when the findings in Experiment 1 after 118 days are compared with those in Experiment 2. They are practically identical. In this experiment, as in the previous one, the most careful sheltering of the mice failed to prevent the occurrence of cancer in a considerable proportion of instances. The comparison just made with Experiment 1 gives every reason for the supposition that if the sheltered animals had been tarred longer or permitted to survive longer, or both, malignancy would have supervened in many more of them.

The spraying with tar of the jars and bedding of the sheltered mice may have been overdone, providing a greater carcinogenetic stimulus than existed in the case of the controls; but the incidence of warts does not suggest this. Nevertheless in the next experiment no tar was provided beyond that placed on the skin. Adult mice habituated to a diet of muscle were employed; and it proved possible to maintain the sheltered and control groups at almost precisely the same weights.

About 200 mice, young adults when first obtained, were kept in boxes of 10 under ordinary conditions for 3 months before two groups of 55 animals were paired from amongst them on the basis of weight and distributed in jars. The average weight for the one group was 29.2 gm. and for the other 29.0 gm. The individual weights were in general within 2 or 3 gm. of this figure. There were 28 male mice in each group and 27 female, none of the latter pregnant. Sex is known to be without influence on the incidence of tar cancer.
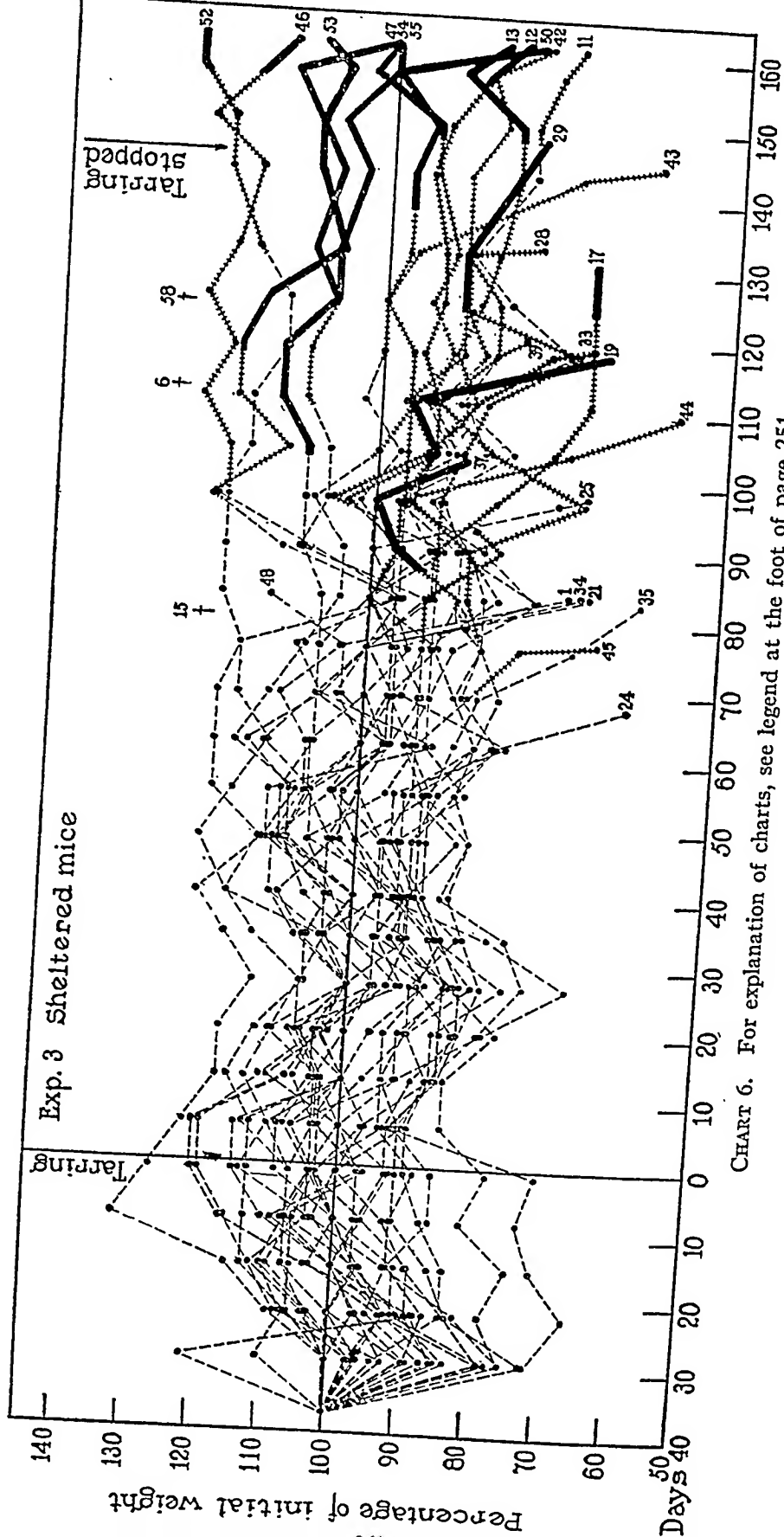
CHART 6. For explanation of charts, see legend at the foot of page 251

Exp. 3 Controls

Tarring

Tarring stopped

CHART 7

Percentage of initial weight

Days

263
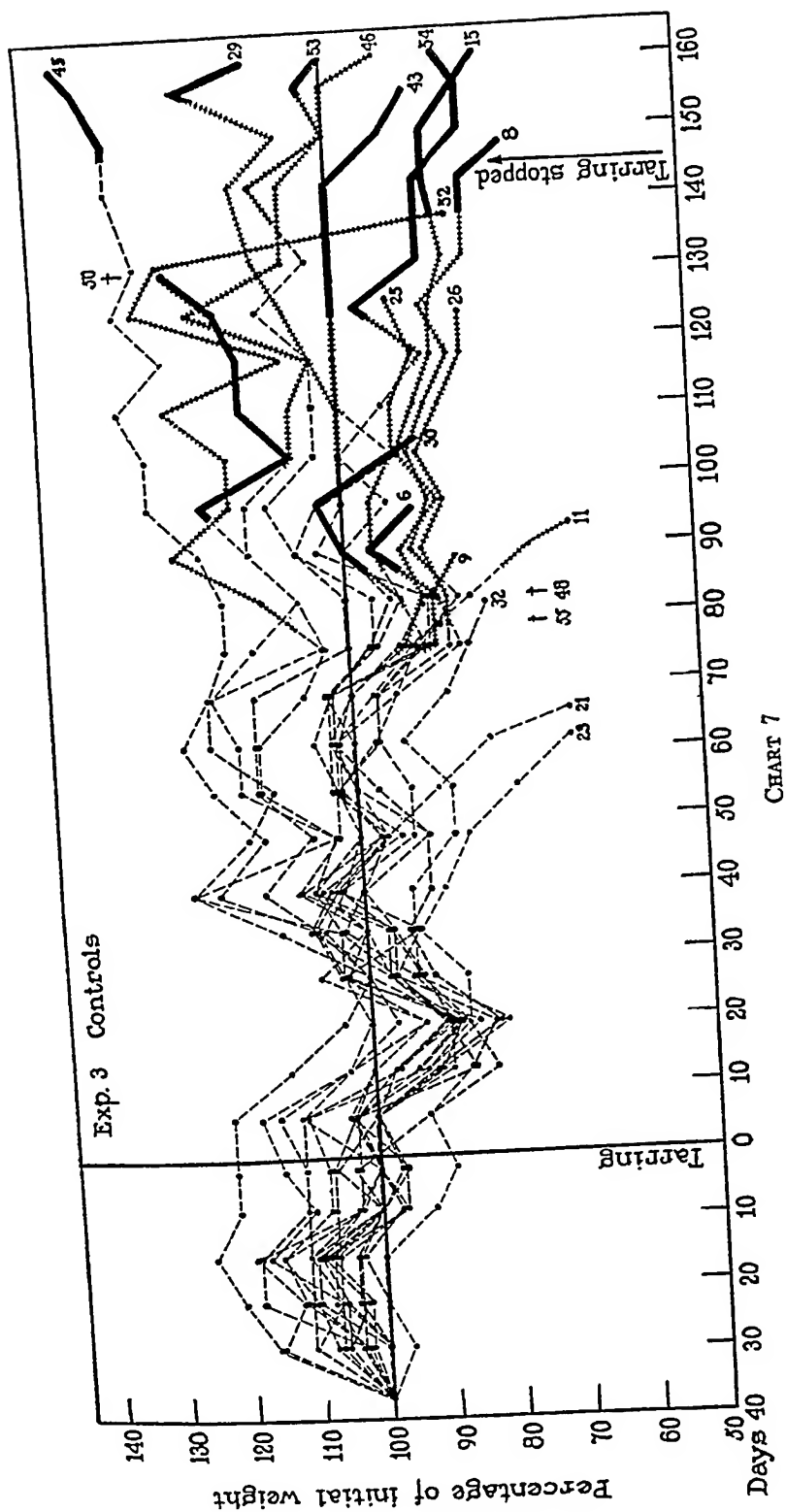
The animals to be sheltered were placed in the same hood as in previous experiments, in a corner of a large, unused laboratory, the controls in an open hood giving onto a dusty room which served as a passage way and in which dogs, guinea pigs and rabbits were almost daily handled. The sterile sawdust of the secluded group was changed only twice a week until the tarrings were begun. So too with the bottles of sterile water.

The muscle employed was fresh lean beef (round steak), in a single large piece procured twice a week, the morning after slaughter. It had been kept on ice overnight. On receipt from the slaughter house it was seethed in boiling water for 3 minutes and placed in the ice box in a sterile container. Just before use it was cut into pieces of approximately 12 gm. with a sterile knife, seethed at 75.0–80.0°C. for 3 minutes, to take the chill off, and distributed with sterile tongs. On the first 9 days of the feeding the seething was in water, thereafter in hot 1.8 per cent sodium chloride solution. The controls received dry food (buckwheat, rolled oats, and chopped dog biscuit), and bread wet with milk, on alternate days. Both series were weighed every week, and the amount of food given the controls was so carefully restricted that they did not outstrip the sheltered group.

After 36 days under these conditions all the mice were shaved, and tarring was begun a day later. The secluded mice had lost some weight to begin with (Chart 6) but recovered it long before the tarrings. Just prior to each of these the sheltered mice were transferred to fresh, sterile shavings and they were handled before the controls, as in the previous experiments. Had the tarring been done on the old bedding some of this latter would have stuck to the skin and been transferred to the new jars.

After 34 days of tarring an epidemic of pneumonia, due, as cultures showed, to *B. friedlaenderi* carried off 13 of the controls within 3 days.[3] No fresh cases occurred after the survivors had been transferred to clean jars. Care was taken for some days thereafter to prevent the carrying over of infectious material from one mouse to another during the distribution of food; but the old conditions were soon instituted again.

The first wart was noted amongst the controls after 75 days of tarring, as compared with 70 days in the secluded group. After 90 days 9 out of 16 controls had developed one or more warts and so had 14 of the 23 secluded animals. In 2 of the controls cancers had also appeared and in 1 of the experimental group. The average weights were 29.6 and 29.1 gm. respectively. The tarring was stopped after 145 days, and the animals were permitted to live 16 days longer without alteration in the conditions of sheltering or exposure. The hair reappeared over the back at the same general rate in both groups, very late, as a scattered thin down. The tar had evidently injured the skin to about the same degree. In 11 of the 12 sheltered animals surviving until the end, one or more cancers had developed, and the remaining mouse was warty. All of the 8 controls carried cancers. Five had already died of the disease and 4 of the experimental animals.

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[3] These animals dying early are not charted.

In this experiment the complicating conditions which had prevented a just comparison in the previous experiments were ruled out. The epidemic of pneumonia attested to the fact that the controls were exposed to intercurrent infection. It carried off principally the ill-nourished mice, those, that is to say, in which cancer was less likely to develop. Nevertheless the incidence of cancer was practically identical in the two groups (Charts 6 and 7). What the charts fail to show is that on the average the warts and cancers of the sheltered and control mice differed not at all in character, rate of growth, and tendency to be multiple.

COMMENT

The fact emerged from our first experiments that sheltering adult mice from the access of living entities by way of food, drink, bedding, and air was insufficient to prevent the development of cancer in a considerable proportion of them, when the appropriate stimulus of tissue injury was provided. It remained for Experiment 3 to show, however, that the incidence of cancer was absolutely unaffected by this precaution. Nor was it influenced in the least by the profound difference in the character of the food given the controls and the sheltered animals. Tarred mice fed exclusively upon raw mammalian muscle became cancerous with precisely the same frequency as those subsisting largely upon cereals. The finding accords with that of Leonard Hill who placed five groups of mice on differing diets and could detect no significant influence of these on the incidence of tar cancer (6).

The periods of 118 to 196 days during which our animals were sheltered comprise from one-sixth to more than one-fourth of the span of life of the mouse, assuming this to be 2 years, a generous estimate. In Experiment 3 the sheltering had lasted for more than 5 weeks before any injury was produced of the sort that ultimately led to cancer. The results prove that mouse cancer cannot be caused by living entities reaching the body from the surrounding world during adult life, and they rule out for this species the possibility of the prevention of the disease by methods based on such an assumption. They fail to exclude the possible activity of entities residing habitually in or upon the body, as do the staphylococci and the colon bacillus.

that hemolysin, inactivated by exposure to air at 38°C. for 6 hours, could be reactivated by reducing substances such as sodium hydrosulfite, but that more prolonged incubation caused irreversible oxidation. They therefore distinguished three degrees of activity of streptolysin: (1) active hemolysin in the fully reduced state, (2) inactive hemolysin which could be reactivated by reduction, (3) inactive hemolysin which was irreversibly oxidised and which could not be reactivated by reducing substances.

Todd (4) showed that streptolysin, obtained from broth cultures containing 20 per cent of normal horse serum, did not react with oxygen in the manner described by Neill and Mallory. The titre of hemolysin in serum broth gradually fell on exposure to incubator temperature and it was found impossible to prevent this decline in activity by exclusion of oxygen. It was also found that inactivation was not accelerated by powerful oxidising agents such as hydrogen peroxide.

The following experiments were done to investigate these discrepancies.

Hemolytic streptococci were grown in broth to which 20 per cent of normal horse serum had been added; and sterile hemolysin was obtained by filtration through a Maasen candle. The hemolysin was titrated immediately after filtration and it was then completely inactivated by storage in a 5 x ¾ inch tube at 25°C. for 2 days. A parallel experiment was performed with a culture of the same strain which had been grown for 16 hours in broth containing 7 per cent of yeast extract instead of serum. In this case inactivation at 25°C. was continued for an extra day to allow more complete oxidation. The two inactive filtrates, which gave the same pH readings, were then reduced with 1.0 per cent of sodium hydrosulfite and phosphate buffer as described by Neill and Mallory (30 minutes at room temperature). Table I shows that the serum-free hemolysin was reactivated to a higher titre than the original filtrate ($Ly_r + Ly_o$ Neill and Mallory); on the other hand sodium hydrosulfite did not cause any return of hemolytic activity in the filtrate which contained serum.

Channon and McLeod (5), working with broth containing 30 per cent of normal ox serum, succeeded in obtaining, by reduction, a slight reactivation of streptolysin which had been inactivated at 37°C. They noted, however, that only a partial reactivation was obtained (M.H.D. of original filtrate = 0.01 cc., M.H.D. of reduced filtrate = 0.5 cc.) in contrast to the experiments of Neill and Mallory who obtained,

by reduction, active hemolysin of equal or higher titre than the original unoxidised fluids prepared from young cultures.

It is evident that streptolysin which contains serum is more resistant to the processes of oxidation and reduction than serum-free hemolysin. Further evidence of this difference is given by the following experiment.

A hemolytic filtrate, prepared from 20 per cent serum broth, was divided into three parts. Part 1 was mixed with $\frac{1}{20}$ of its volume of hydrogen peroxide, Part

TABLE I

Showing the Effect of Reduction by Sodium Hydrosulfite on Yeast Extract Streptolysin and on Serum Streptolysin Which Had Both Been Inactivated by Storage at 25°C.

Volume of hemolysin	Hemolysin prepared with yeast extract			Hemolysin prepared with normal horse serum		
	Original filtrate	Filtrate inactivated by storage for 3 days at 25°C.	Inactivated filtrate after reduction with Na₂S₂O₄	Original filtrate	Filtrate inactivated by storage for 2 days at 25°C.	Inactivated filtrate after reduction with Na₂S₂O₄
cc.						
0.5						
0.2	+	0	+++	+++	0	0
0.1	+	0	+	+++	0	0
0.05	+	0	+	+++	0	0
0.02	0	0	+	+++	0	0
0.01	0	0	+	++	0	0
0.005	−	−	0	+	0	0
0.002	−	−	−	+	0	0
0.001	−	−	−	+	0	0
				0	0	0

Control: Broth + 1 per cent Na₂S₂O₄ = no hemolysis. +++ = complete hemolysis. ++ = incomplete hemolysis. + = partial hemolysis. 0 = absence of hemolysis. − = not tested.

2 received the same volume of a mixture of hydrogen peroxide and peroxidase; and the volume of the third part was adjusted with normal saline. After 24 hours at room temperature the three preparations were equal in hemolytic titre. In a parallel experiment, with serum-free hemolysin, the peroxide preparations were less active than the control after 3 hours at room temperature and after 24 hours they were completely inactivated while the control retained a part of its original activity.

Hemolysin in serum broth may also be distinguished from serum-free hemolysin by the following characteristic differences.

antigen. Evidence that the filtrate remained slightly antigenic after heating at 100°C. is derived from the observation that 1.0 cc. of serum, from the rabbit which received boiled antigen, neutralised increasingly large fractions of the test dose of hemolysin; during the first 4 weeks 1.0 cc. of serum allowed complete hemolysis; during the 8th, 9th, and 10th weeks 1.0 cc. of serum allowed only a trace of hemolysis; and

TABLE II

Showing the Relative Neutralising Values of Active Yeast Extract Streptolysin and of Streptolysin Inactivated at Various Temperatures on Antistreptolysin

Volume of serum	Antihemolytic titre of immune serum after 4 days incubation with				
	Active reduced hemolysin	Hemolysin inactivated at 37°C.	Hemolysin inactivated at 60°C.	Hemolysin inactivated at 100°C.	Normal saline
cc.					
0.005	0	0	—	—	—
0.004	0	0	—	—	—
0.003	0	0	—	—	—
0.002	0	0	—	—	—
0.001	+	0	0	0	0
0.0009	++	0	0	0	0
0.0008	++	+	0	0	0
0.0007	+++	+	0	0	0
0.0006	+++	+	0	0	0
0.0005	+++	++	+	0	0
0.0004	+++	++	+	+	0
0.0003	+++	+++	++	+	+
0.0002	+++	+++	++	++	++
0.0001	+++	+++	+++	+++	+++
Saline control	+++	+++	+++	+++	+++

+++ = complete hemolysis.   ++ = incomplete hemolysis.   + = partial hemolysis.   0 = absence of hemolysis.   — = not tested.

after the 12th week, when inoculations had been stopped for 3 weeks, 1.0 cc. of serum again allowed complete hemolysis. There was, therefore, a definite increase in antihemolysin due to immunisation with boiled antigen, although the serum did not at any time contain sufficient antihemolysin in 1.0 cc. to neutralise completely $2\frac{1}{2}$ M.H.D. of hemolysin. This effect of boiled antigen, which was not accurately measured, is represented by the dotted line in Fig. 1.

In order to exclude the possibility that these differences in response

to inoculation were due to individual differences in the reactive capacity of the rabbits, an experiment, similar to an absorption test, was set up with the four antigens used for immunisation.

Five sets of equally graded dilutions of high titre antihemolytic horse serum were made in 0.1 cc. volumes; 0.5 cc. of active reduced hemolysin was added to each tube of the first set of dilutions; three sets of dilutions were respectively treated with 0.5 cc. of hemolysin irreversibly oxidised at 37°C., hemolysin inactivated at 60°C., and hemolysin inactivated at 100°C.; and the fifth set of dilutions was used as a control by substituting normal saline for antigen. All the tubes were incubated at 37°C. and an additional series of tubes each containing 0.1 cc. of saline, instead of serum, and 0.5 cc. of active hemolysin was included in the experiment for periodical observations on the activity of the hemolysin. After 24 hours incubation the hemolysin was inactive and after 4 days it was irreversibly oxidised. The five sets of tubes were therefore removed from the incubator on the 4th day and their antihemolysin content was determined in the usual way (see Appendix 3). The result of this experiment is shown in Table II.

The original dose of serum which completely neutralised $2\frac{1}{2}$ M.H.D. of hemolysin was 0.0004 cc. and the titre of the serum remained unchanged after incubation with normal saline for 4 days. On the other hand all the antigens reduced the titre of the serum; the figures given below are the differences in antihemolytic titre between untreated serum and serum absorbed with the specified antigens. Hemolysin absorbed the antihemolysin as follows:

Active hemolysin absorbed antihemolysin in 0.0016 cc. serum (0.002 −0.0004).
Inactivated at 37°C.   "   "   " 0.0005 "   "   (0.0009−0.0004).
   "   "   60°C.   "   "   " 0.0002 "   "   (0.0006−0.0004).
   "   " 100°C.   "   "   " 0.0001 "   "   (0.0005−0.0004).

Active hemolysin absorbed the largest quantity of antihemolysin and the power of each antigen to absorb antihemolysin corresponded with its activity as an immunising agent for rabbits.

Antihemolysin in Scarlet Fever Antitoxin

Fig. 1 and Table II show that the long exposure to high temperature required to destroy the antigenic activity of yeast extract streptolysin is of the same order as the temperature conditions required to destroy the skin reactivity of Dick toxin. This observation suggested an examination of scarlet fever antitoxin for antihemolysin.

Samples of scarlet fever antitoxin, puerperal fever antitoxin, and erysipelas antitoxin were tested for antistreptolysin by the method described in Appendix 3; they all contained considerable quantities of antihemolysin. The specificity of

antigen. Evidence that the filtrate remained slightly antigenic after heating at 100°C. is derived from the observation that 1.0 cc. of serum, from the rabbit which received boiled antigen, neutralised increasingly large fractions of the test dose of hemolysin; during the first 4 weeks 1.0 cc. of serum allowed complete hemolysis; during the 8th, 9th, and 10th weeks 1.0 cc. of serum allowed only a trace of hemolysis; and

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cc.					
0.005	0	0	—	—	—
0.004	0	0	—	—	—
0.003	0	0	—	—	—
0.002	0	0	—	—	—
0.001	+	0	0	0	0
0.0009	++	0	0	0	0
0.0008	++	+	0	0	0
0.0007	+++	+	0	0	0
0.0006	+++	+	0	0	0
0.0005	+++	++	+	0	0
0.0004	+++	++	+	+	0
0.0003	+++	+++	++	+	+
0.0002	+++	+++	++	++	++
0.0001	+++	+++	+++	+++	+++
Saline control	+++	+++	+++	+++	+++

+++ = complete hemolysis. ++ = incomplete hemolysis. + = partial hemolysis. 0 = absence of hemolysis. — = not tested.

after the 12th week, when inoculations had been stopped for 3 weeks, 1.0 cc. of serum again allowed complete hemolysis. There was, therefore, a definite increase in antihemolysin due to immunisation with boiled antigen, although the serum did not at any time contain sufficient antihemolysin in 1.0 cc. to neutralise completely $2\frac{1}{2}$ M.H.D. of hemolysin. This effect of boiled antigen, which was not accurately measured, is represented by the dotted line in Fig. 1.

In order to exclude the possibility that these differences in response

antitoxin prepared from non-hemolytic streptococci, and antibacterial sera prepared from pneumococci, meningococci, and *B. welchii*. The source, the antitoxic value, and the antistreptolysin content of the sera tested are given in Table III; the neutralising doses of sera from horses immunised with hemolytic streptococcal toxins ranged from 0.0009 to 0.0002 cc. while the doses of control sera ranged from 0.4 to 0.02 cc.

Comparative Effects of Scarlet Fever Antitoxin on Serum Streptolysin and on Yeast Extract Streptolysin

In view of the reported failures to detect large quantities of antihemolysin in the serum of immunised animals, scarlet fever antitoxin and normal horse serum were both titrated against $2\frac{1}{2}$ M.H.D. of yeast extract streptolysin and also against $2\frac{1}{2}$ M.H.D. of serum streptolysin.

0.0003 cc. antitoxin	neutralised	$2\frac{1}{2}$ M.H.D yeast extract hemolysin.
0.05 " normal serum	"	$2\frac{1}{2}$ " " " "
0.1 " antitoxin	failed to neutralise $2\frac{1}{2}$ " serum hemolysin.
0.1 " normal serum	" " "	$2\frac{1}{2}$ " " "

An exact determination of the dose of antitoxin required to neutralise serum hemolysin was impossible, in this experiment, owing to hemolysis by the preservative in undiluted antitoxin. A similar experiment was therefore done with fresh sera (*a*) from a normal horse, (*b*) from a horse partially immunised with scarlet fever toxin.

0.005 cc. immune serum neutralised $2\frac{1}{2}$ M.H.D. yeast extract streptolysin.
0.05 " normal " "	$2\frac{1}{2}$ " " " "
0.5 " immune " "	1 " serum streptolysin.
0.5 " normal " "	1 " " "

The immune serum was ten times stronger than the normal serum in its power to neutralise yeast extract hemolysin, and yet the two sera were equal in their ability to neutralise serum hemolysin. This suggests that failure to detect any increase of antistreptolysin in the sera of animals immunised with streptococcal filtrates has been due to methods of testing for antihemolysin rather than to methods of immunisation.

DISCUSSION

Before Neill and Mallory (2) discovered the effect of yeast extract on the production and preservation of streptolysin, and before they demonstrated the extreme sensitiveness of this form of hemolysin to oxidation, it was impossible to prepare sterile streptolysin, of sufficiently

TABLE III

Antistreptolysin Content of Horse Sera

Description of sera	Dose of serum required to neutralise 2½ M.H.D. of streptolysin
	cc.
Normal	0.04
"	0.03
"	0.3
"	0.2
"	0.05
"	0.03
Scarlet fever antitoxin	0.0003
" " "	0.0003
" " "	0.0002
" " "	0.0007
" " "	0.0009
" " " (concentrated) 400 units per cc	0.0003
" " " Hygienic Laboratory Washington, Standard, 40 units per cc	0.005
Erysipelas antitoxic and antibacterial (concentrated)	0.0006
Puerperal septicemia antitoxic and antibacterial (concentrated)	0.0003
Diphtheria antitoxin 1,000 units per cc	0.2
" " 1,500 " " "	0.04
" " 2,300 " " "	0.03
Tetanus antitoxin 2,400 international units per cc	0.06
" " 2,400 " " " "	0.02
" " 1,500 " " " "	0.04
" " 1,200 " " " "	0.02
Staphylococcus antitoxin 1/1,000 neutralised 8 M.H.D.	0.02
" " 1/1,000 " 8 "	0.02
Small's non-hemolytic streptococcus antitoxin	0.2
Antipneumococcus 100 Felton units per cc	0.2
" 100 " " " "	0.3
* Antimeningococcus. Agglutination titre 1 in 250	0.4
* " " " 1 in 25	0.2
* " " " 1 in 25	0.02
Anti-gas-gangrene (*B. welchii*). 0 agglutination 1 in 5,000	0.09

All sera unconcentrated unless otherwise stated.

* From horses partially immunised.

streptococcal antihemolysin is shown by the low titre of antistreptolysin in normal horse serum and in the various immune horse sera tested, which included antisera to the hemolytic toxins of staphylococci, tetanus bacilli, and diphtheria bacilli,

antitoxin prepared from non-hemolytic streptococci, and antibacterial sera prepared from pneumococci, meningococci, and *B. welchii*. The source, the antitoxic value, and the antistreptolysin content of the sera tested are given in Table III; the neutralising doses of sera from horses immunised with hemolytic streptococcal toxins ranged from 0.0009 to 0.0002 cc. while the doses of control sera ranged from 0.4 to 0.02 cc.

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0.0003 cc. antitoxin	neutralised	$2\frac{1}{2}$ M.H.D yeast extract hemolysin.			
0.05 " normal serum	"	$2\frac{1}{2}$ " " " "
0.1 " antitoxin	failed to neutralise $2\frac{1}{2}$ " serum hemolysin.
0.1 " normal serum	" " " $2\frac{1}{2}$ " " "

An exact determination of the dose of antitoxin required to neutralise serum hemolysin was impossible, in this experiment, owing to hemolysis by the preservative in undiluted antitoxin. A similar experiment was therefore done with fresh sera (a) from a normal horse, (b) from a horse partially immunised with scarlet fever toxin.

0.005 cc. immune serum neutralised $2\frac{1}{2}$ M.H.D. yeast extract streptolysin.					
0.05 " normal " " $2\frac{1}{2}$ " " " "
0.5 " immune " " 1 " serum streptolysin.
0.5 " normal " " 1 " " "

The immune serum was ten times stronger than the normal serum in its power to neutralise yeast extract hemolysin, and yet the two sera were equal in their ability to neutralise serum hemolysin. This suggests that failure to detect any increase of antistreptolysin in the sera of animals immunised with streptococcal filtrates has been due to methods of testing for antihemolysin rather than to methods of immunisation.

DISCUSSION

Before Neill and Mallory (2) discovered the effect of yeast extract on the production and preservation of streptolysin, and before they demonstrated the extreme sensitiveness of this form of hemolysin to oxidation, it was impossible to prepare sterile streptolysin, of sufficiently

high titre to be useful in laboratory tests, without adding large quantities of normal serum to the broth. Experiments recorded in this paper show that serum affects hemolysin in unexpected ways, causing delayed hemolysis, resistance to oxidation or reduction, and inability to combine with the specific antihemolysin which readily neutralises yeast extract streptolysin. An experiment has been quoted showing that although an immune serum was ten times more active than a normal serum in neutralising yeast extract hemolysin, yet these two sera were equal in their ability to neutralise serum hemolysin. It therefore seems probable that the neutralisation of serum streptolysin by normal and immune sera is dependent on the chemical composition of the sera and is not due to the action of a specific antibody. This hypothesis is supported by the observations of Lyall (6) who, working with guinea pig, rabbit, cow, human, and sheep sera showed that, with occasional variations, the volume of serum required to neutralise a unit of serum hemolysin is uniform for the same species of animal. McLeod and McNee (7) showed that rabbits become anemic when injected with serum streptolysin and that repeated injections not only fail to cause immunity but actually render relatively insusceptible rabbits more susceptible to serum hemolysin. This observation is in marked contrast to the experiments on immunity to yeast extract streptolysin recorded in this paper which show that repeated injections of active yeast extract streptolysin produce only transitory toxic symptoms in rabbits and stimulate the production of specific antihemolysin. It is also possible to demonstrate that animals immunised with yeast extract streptolysin are more resistant to infection by the relatively attenuated variants of hemolytic streptococci than normal animals, although their immunity to highly virulent variants is not increased. These important differences between the properties of two substances which possess certain common characteristics, but which are obviously not identical, necessitate consideration of the relationship which presumably exists between serum hemolysin and yeast extract hemolysin. It is possible that serum streptolysin owes its distinctive characteristics, namely filterability, relatively slow lysis, resistance to oxidation or reduction, and absence of antigenicity, to adsorption by serum; and that the free streptolysin which is formed in cultures without serum owes its greater lability and activity to the absence of any adsorbing substance.

The use of yeast extract streptolysin for quantitative estimations of antihemolysin has shown that there is considerable variation in the quantity of specific antistreptolysin in the sera of normal animals; this variation possibly depends on the susceptibility of the species to infection by hemolytic streptococci. Estimations of the specific anti-streptolysin in the sera of apparently normal men and horses, both notoriously subject to attack by hemolytic streptococci, show that the neutralising dose of serum may vary from 0.3 to 0.02 cc.; on the other hand sera of normal rabbits which seldom, if ever, suffer from natural infection with non-capsulated hemolytic streptococci, have a uni-formly low antistreptolysin titre. It seems probable that the varia-tions in specific antistreptolysin in human and in horse sera may be due to previous infections and to subinfections with hemolytic strep-tococci. Observations have been made on sera from patients infected with hemolytic streptococci and, although the numbers are at present small, yet all the cases which have recovered from the infection have developed increasing quantities of specific antistreptolysin in their sera during the course of the illness.

Table III shows that horses immunised with the toxins of hemolytic streptococci derived from cases of scarlet fever, erysipelas, and puer-peral fever all develop specific antistreptolysin capable of neutralising yeast extract hemolysin prepared from a scarlet fever strain. Con-versely antihemolysin, prepared by immunising a horse with filtrate from one serological type of scarlet fever streptococcus, neutralises the streptolysin of both homologous and heterologous strains to the same titre. It therefore seems probable that the hemolysins of all streptococci are serologically identical; and if, after more extensive observations, this is found to be the case there are a number of prob-lems which may be elucidated by observations on specific antistreptoly-sin in sera. A constant rise of antistreptolysin in the serum, during the course of a disease of uncertain etiology, may be taken as evidence that the disease is associated with infection by hemolytic streptococci; it is hoped that this test may be useful in the study of rheumatic fever. In diseases which are certainly caused by hemolytic strepto-cocci, such as puerperal septicemia, observations on the rise or fall of immunity to streptolysin may be significant in prognosis and may throw light on the patients' reaction to infection.

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Observations on specific antistreptolysin in the sera of horses under-going immunisation with scarlet fever toxin may possibly be used to gauge the antitoxic value of the serum; preliminary observations show that there is some correlation between antitoxin and antihemolysin but whether they are distinct and separate or identical antibodies is at present uncertain. The relationship between serum-free streptolysin and skin-reactive Dick toxin is also a subject for further investigation.

SUMMARY

1. Normal serum, used in cultures for preparation of the streptolysin, modifies the properties of the streptolysin, causing delayed hemolysis, increased filterability, resistance to oxidation or reduction, and absence of antigenicity.

1. Streptolysin prepared without serum is an active antigen.

3. Similar temperatures are required to destroy the antigenic activity of serum-free streptolysin and the skin reactivity of Dick toxin.

4. Scarlet fever antitoxin contains antistreptolysin which does not neutralise serum streptolysin and which can be detected only by titration against serum-free streptolysin.

5. The antihemolysin which neutralises serum-free streptolysin is species-specific but not type-specific.

Appendix 1. Preparation of Yeast Extract

Thjötta and Avery prepared yeast extract by adding 100 gm. of brewer's yeast to 400 cc. distilled water; pH adjusted to 4.6; boiled over flame for 10 minutes; sedimented at room temperature; supernatant tested for sterility; stored in cold at pH 4.6; reaction adjusted as required immediately before use. In experiments now recorded yeast grown in the laboratory gave better results than brewer's yeast. The following methods were devised by Mr. J. H. Worker. Dissolve 2 per cent of agar in beer wort in steamer; fill into Roux bottles, sterilise by steaming for 45 minutes on 3 successive days and allow to solidify in incubator to ensure level surfaces of medium for inoculation with yeast. Incubate *Saccharomyces cerevisiae* for 2 days on beer wort agar slopes in 7 x 1½ inch tubes, wash off in 15 cc. saline, and use washings to inoculate bottles (5 cc. to 7 x 4 inch bottles containing 70 cc. of medium). Incubate bottles for 7 days at 25° to 30°C. Wash off yeast with sterile distilled water, centrifuge down, discard supernatant, weigh

yeast, and emulsify in distilled water (25 gm. to 100 cc.), boil as previously described. The pH is usually about 4.5, and adjustment of reaction before boiling is therefore unnecessary.

Appendix 2. Preparation of Active Reduced Streptolysin (Antigenic)

Neill and Mallory used a special "unfiltered" muscle infusion broth for preparing serum-free hemolysin. In experiments now recorded a number of different types of broth have been used including "unfiltered" muscle infusion broth, tryptic digest broth, beef and veal infusion broth, and horse flesh infusion broth. The best results have been obtained with horse flesh infusion broth containing 2 per cent Difco proteose peptone, adjusted to pH 8.0; but there is great variation in the yield of streptolysin from different batches of broth prepared in the same way and, as the essential factor responsible for this variation in streptolysin production is unknown, broth which is satisfactory for streptolysin production has been secured by testing every batch of broth, made for the routine work of the laboratory, and reserving suitable batches for streptolysin production. The broth is sterilised by filtration and distributed in 93 cc. volumes in 7 x 1½ inch tubes, autoclaved at 115°C. for 5 minutes with an equal number of tubes containing 15 cc. of vaseline. When the autoclave is opened the hot vaseline is poured on to the surface of the medium and the broth is stored at room temperature.

Addition of Yeast Extract.—7 cc. of yeast extract (see Appendix 1) is adjusted to pH 8.0, placed under negative pressure to remove dissolved air, and added to broth after melting the vaseline seal. The medium is mixed by inverting the tube after the vaseline seal has solidified.

Inoculation of Culture.—Three tubes of yeast extract broth, each containing 100 cc. of medium are sown with a suitable culture of hemolytic streptococcus by piercing the vaseline and introducing 0.2 cc. of a 6 hour broth culture with a capillary pipette. After resealing with vaseline the culture is incubated for 16 hours at 37°C.

Filtration.—A 5 x ¾ inch Maasen candle is especially prepared to prevent adsorption of hemolysin by immersion for 16 hours in fresh normal horse serum which is then removed by filtration; excess of serum, in the pores of the candle, is washed out by filtration of normal saline. Cultures are then filtered through the candle by negative pressure.

Reduction of Streptolysin.—The filtrate is reduced by adding 3.0 cc. N NaOH and 1.5 gm. of sodium hydrosulfite to 300 cc. of filtrate in the filter flask which is closed with a rubber bung and exhausted with a water pump for ½ hour at room temperature. The pH is then adjusted to give a pink colour with phenol red on a white tile; accurate determination of pH is difficult as the sodium hydrosulfite rapidly decolourises the dye (7.5 cc. of N NaOH is usually added to 300 cc. of filtrate). The reduced filtrate is stored in the refrigerator in 12 cc. volumes in narrow tubes sealed with vaseline; and, under these conditions, the hemolytic activity is maintained for several weeks. Reduced filtrate made up to 0.5 cc. with normal saline and incubated with 0.5 cc. of 5 per cent washed rabbit cells in

a water bath at 37°C. for 1 hour usually causes hemolysis as follows:—0.5 and 0.2 cc. complete hemolysis; 0.1 and 0.05 cc. almost complete; 0.02 cc. marked; 0.01 cc. trace; 0.005 cc. no hemolysis.

Appendix 3.   Titration of Specific Antistreptolysin in Serum

The serum to be tested is inactivated for 15 minutes at 56°C. to prevent hemolysis by natural cytolysin; inactivation is unnecessary if the serum is known to contain a large quantity of antistreptolysin as hemolysis by cytolysin only occurs in low dilutions of serum.   A preliminary titration is made with tenfold dilutions of serum to ascertain the approximate titre of antistreptolysin; and the exact titre is determined by a second titration with closely spaced dilutions.

Preliminary Titration.—The serum is diluted 1 in 10, 1 in 100, 1 in 1,000, and 1 in 10,000 in normal saline; 1.0 cc. of each dilution is transferred to a series of 3 x ¾ inch tubes; 2½ M.H.D. (usually 0.5 cc.) of reduced yeast extract streptolysin is added to each tube; the tubes are shaken and then incubated for 15 minutes in a water bath at 37°C. to allow neutralisation of streptolysin.   The dose of serum which has neutralised the test dose of streptolysin is determined by adding 0.5 cc. of 5 per cent washed rabbit cells in normal saline, shaking, returning to water bath, shaking again after 15 minutes incubation, and reading the result after 1 hour at 37°C.   The cells are finally centrifuged down to detect traces of hemolysis.

Final Titration.—The exact titre of antistreptolysin is determined by testing intermediate dilutions in the same way, *e.g.* if the preliminary titration gives no hemolysis with 0.001 cc. of serum and partial or complete hemolysis with 0.0001 cc. of serum, ten intermediate dilutions in 1.0 cc. volumes are made; *i.e.*, 0.001 cc., 0.0009 cc., 0.0008 cc., etc., to 0.0001 cc.   These dilutions are titrated against 2½ M.H.D. of streptolysin from a freshly opened tube and the smallest volume of serum which completely neutralises 2½ M.H.D. of streptolysin is taken as the final reading.

Determination of the highest dilution in which complete hemolysis occurs is only an approximate test of the activity of yeast extract streptolysin since the occurrence of complete or incomplete hemolysis is dependent on the time which elapses between the addition of red cells and their intimate mixture with the streptolysin.

As it is impossible to titrate hemolysin with sufficient accuracy to ensure absolute equality of the test doses of different batches of streptolysin it is essential to include a standard serum of known antistreptolysin content in each series of tests.

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ON THE NUTRITIONAL REQUIREMENTS ~~vITRO OF~~ NORMAL AND MALIGNANT MOUSE EPITHELIUMS

By LARS SANTESSON

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 14 TO 16

In order to gain a better knowledge of the fundamental properties of normal and malignant tissues, it is desirable to study *in vitro* various cell types obtained from normal tissues and from experimental and spontaneous tumors. In this manner, normal and malignant tissues can be accurately compared. Such a comparison must be made on pure cultures and between cells from the same animal species. Up to now, it has not been possible to compare the only malignant epithelium of the mouse yet obtained in pure culture, that is, the epithelium from Ehrlich carcinoma,[1] with less malignant or with normal homologous epitheliums, since the techniques necessary for obtaining pure strains of mouse tissues have still to be discovered. The purpose of the present paper is to describe some relations between the nutritional requirements *in vitro* of epithelium derived from normal organs of adult mice and those of epithelium from spontaneous and transplanted mammary gland tumors.

EXPERIMENTAL

Normal Tissues.—The mammary gland is embedded in fat and composed of only a few epithelial tubules. Therefore, the probability of cutting through epithelial tissue, when preparing fragments for cultivation, is small. Even in suitable media, epithelial migration does not regularly occur. It is necessary to make a considerable number of explants from each gland in order to obtain a few results. Epithelial migration occurred most frequently in cultures of glands of animals in early pregnancy, whereas epithelial cells from glands of

[1] Fischer, A., Z. Krebsforsch., 1927, **25**, 89.

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non-pregnant animals generally failed to migrate. Explants from mammary glands of twenty-nine adult mice[2] have been cultivated, at least fifteen explants of each being made. Other experiments were made with adult mouse organs whose anatomical structure does not hinder epithelial migration. Kidney, liver, urinary bladder, and salivary glands were studied. At least five explants from each organ were made.

Tumor Tissues.—Spontaneous epithelial mammary gland tumors, 86 in number, were cultivated. They belonged to the adenocarcinomatous type. Cultures were likewise obtained from explants derived from twenty-seven transplanted tumors of Ehrlich mouse carcinoma. These epithelial tumors consisted of structures originally arising from the ducts of the normal mammary gland, and possessed only a small amount of stroma. In suitable media, a large epithelial migration extended from the fragments.

Techniques.—Identical techniques were employed for both normal and malignant epithelial tissues. The tissues were cultivated according to the hanging drop and the flask procedures. The solid phase of the medium was composed of chicken, rat, or mouse plasma, either pure or mixed together in various proportions. As nutritive phase, sera and embryonic extracts from chicken, mouse, and rat, and extracts of adult mouse organs (spleen, lung, liver, and placenta) were used. The techniques for obtaining plasma, embryonic extracts, and sera were the usual ones Rat and mouse plasmas were secured with the aid of heparin. The organs were sliced with scissors, and to the pulp approximately its own volume of Tyrode solution was added. After a few hours, the mixture was centrifuged, and the supernatant fluid was removed and used either pure or diluted with Tyrode solution. The various extracts were kept in a refrigerator and always used within a week after they had been prepared. When media that could be secured in small amounts were employed, such as mouse serum and plasma, and extracts of mouse placenta, the hanging drop technique was exclusively applied. Otherwise, the cultivation was carried out both in hanging drops and in Carrel flasks (Type D-3.5). The hanging drop cultures were transferred every 2nd or 3rd day. The flask cultures were treated according to the media and tissues used.

The tissues, after removal from the animal, were cut into small pieces about 0.5 to 1.0 mm. long, and washed in Tyrode solution. The rate of migration was measured according to the method of Carrel.[3]

Various Types of Supporting Media.—Preliminary investigations were carried out to find the most suitable plasma coagulum for sup-

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[2] Of these, 22 were pregnant animals.
[3] Carrel, A., *J. Exp. Med.*, 1923, 38, 407.

porting tissues. Mouse, rat, and chicken plasmas, either pure or mixed in different proportions, were used as a hanging drop. It was found that mouse and rat coagula were readily attacked by all cell types. Media composed of chicken plasma were less rapidly liquefied. The former, on the other hand, invariably yielded better and more ready epithelial migration than the latter. Similar results were obtained with the flask technique. As solid phase, 0.5 to 1.5 cc. of rat and chicken plasma, diluted 1:2 to 1:8 with Tyrode solution and coagulated by 2 drops of chick embryonic juice, was tested either singly or when mixed together in various proportions. The liquefaction of the plasma clot was the more marked, the more rat plasma the medium contained. After a short time in rat plasma, the cultures were almost always found floating in a pool of fluid which prevented further cell migration. Clots consisting of diluted chicken plasma alone were less readily attacked. On the other hand, they seemed to inhibit the activity of the epithelial cells. According to Carrel,[4] however, chicken plasma, when it is diluted with 3 volumes of Tyrode solution, coagulated with 2 drops of chick embryonic juice, and then washed once or several times in an excess of Tyrode solution, yields a coagulum that is not toxic for foreign cells. Clots of chicken plasma treated in this way did not inhibit cell activity. In many cases, however, such coagula were liquefied by the cells, although always to a much less extent than the other solid media tested.[5]

As no better supporting medium was found, the following procedure was regularly employed: 1.0 cc. of chicken plasma was diluted 1:4 to 1:8 with Tyrode solution, coagulated with 2 drops of chick embryonic juice, and washed twice for $\frac{1}{2}$ hour in an excess of Tyrode solution (3.00 cc.). After 24 hours, and later according to the amount of liquefaction occurring, the clot was reinforced with 0.5 cc. of the same plasma mixture.

General Characteristics of the Cultures.—(Figs. 1–3.) Pure epithelial cells, or epithelial cells mixed with a few ameboid cells and fibroblasts, migrated from the explants. Each of the three cell types exhibited the appearance found in tissues from other animals. The epithelial cells

[4] Carrel, A., *Compt. rend. Soc. biol.*, 1927, 96, 601.
[5] Variations in this property according to differences in type of tumor cells will be dealt with in a subsequent article.

expanded in regular, thin sheets composed of healthy, uniform cells in pavement-like order.[6]   The number of fibroblasts and ameboid cells varied widely according to the type of explant and to the various media tested.   Whichever nutritive medium was employed, these cell types, even if at first abundant, did not manifest greater or longer activity than epithelial cells.   When derived from explants of normal tissues, they never had any tendency to overgrow the epithelium. Thus, they acted in a manner opposite to that of chicken fibroblasts and epithelium when growing together.[7,8]   Mixed cultures generally yielded pure epithelium after one or two transfers.   Membranes of mouse epithelium were easily obtained, while pure strains of epithelial cells were difficult to isolate from contaminated cultures of chicken tissues.

Comparative Action of Various Nutritive Media.—The following fluids, either pure or mixed, or diluted with Tyrode solution, were studied: sera from chickens, rats, and mice; extracts from embryos of these animals; and extracts of adult mouse organs (lung, liver, and spleen).   Since none of the various nutritive media tested allowed active, continued migration of mouse tissues except of epithelium from Ehrlich carcinoma, no accurate measurements of the rate of cell migration could be made.   The comparative investigations on the action of the nutritive media were, therefore, limited to the study of the epithelial activity occurring in the original culture and of its duration.

The results of the study of cell activity in the original cultures are summarized in Table I.   They are based on the comparison of the extent of epithelial migration in the various media.   Therefore, only obvious differences are recorded.   As may be seen in the table, there is a close similarity between the action of the various nutritive media on the epithelial migration of normal and of tumor epitheliums. Whichever nutritive medium is employed, no significant difference appears in the behavior of the different tissues.

Tyrode Solution.—With Tyrode solution alone, epithelial activity varied. The explants of normal tissues and of most spontaneous tumors did not manifest

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[6] Variations in the architecture of the cultures, and in cell morphology as well as in the rate and duration of migration, will be dealt with in a subsequent article.

[7] Kapel, O., *Arch. Zellforsch.*, 1929, 8, 35.

[8] Fischer, A., *J. Exp. Med.*, 1922, 35, 367.

TABLE I

Effect of Various Nutritive Media on Mouse Epitheliums

Type of tissue	Tyrode solution	Sera			Embryo extracts			Mouse organ extracts		
		Chicken	Mouse	Rat	Chicken	Mouse	Rat	Lung	Spleen	Liver
Normal mammary gland..	0*	0	+ to ++	+++	+	+	+	0 to +.	0	0
Spontaneous epithelial mammary gland tumors..	0 to +									
Ehrlich carcinoma..	+	0	++	++ to +++ ++	++ to ++ ++ to +++ +	+ to ++ ++ to +++	+ to ++	+ to ++ ++		
Adult kidney epithelium..	0	0 to +	++	+++ +++					0	0 to +
Epithelium from urinary bladder..	0			+++	+					

*0 to + to ++++ indicates amount of epithelial activity.

expanded in regular, thin sheets composed of healthy, uniform cells in pavement-like order.[6]   The number of fibroblasts and ameboid cells varied widely according to the type of explant and to the various media tested.   Whichever nutritive medium was employed, these cell types, even if at first abundant, did not manifest greater or longer activity than epithelial cells.   When derived from explants of normal tissues, they never had any tendency to overgrow the epithelium. Thus, they acted in a manner opposite to that of chicken fibroblasts and epithelium when growing together.[7,8]   Mixed cultures generally yielded pure epithelium after one or two transfers.   Membranes of mouse epithelium were easily obtained, while pure strains of epithelial cells were difficult to isolate from contaminated cultures of chicken tissues.

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Spontaneous epithelial mammary gland tumors	0 to +	0 to +	++	++ to ++++	+ to ++	+ to ++	+ to +++	+ to ++	0	0 to +
Ehrlich carcinoma	+		++	+++	++ to ++++	++ to +++	++	++		
Adult kidney epithelium	0			+++	+					
Epithelium from urinary bladder	0			+++	+					

*0 to + to ++++ indicates amount of epithelial activity.

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any activity at all. In the explants from Ehrlich carcinoma and from spontaneous tumors, slow epithelial migration occurred, which soon ceased. It was less extensive than in tissue extracts or blood serum, with the exception of chicken serum.

Blood Serum.—In chicken serum, epithelium, fibroblasts, and ameboid cells remained inactive. This fluid completely inhibited these cells. Mouse serum tested in hanging drops gave less epithelial growth than rat serum. From the beginning of cultivation, cells of granular appearance were observed. Furthermore, the cultures degenerated and ceased to migrate much earlier than those in rat serum. In Table I, the superiority of rat serum as a nutritive medium is clearly shown. In this fluid diluted with 50 per cent Tyroide solution, all ex-
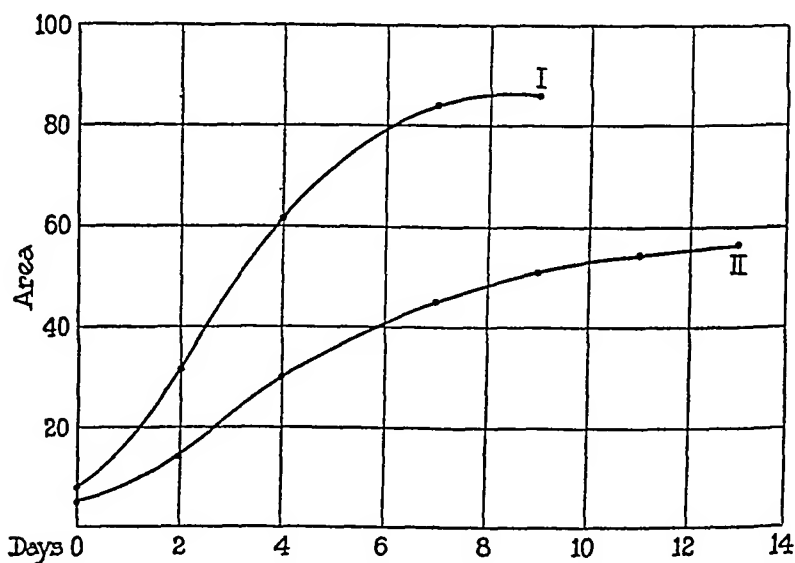
Text-Fig. 1. Experiment 475 S. Effect of 50 per cent rat serum on epithelium from spontaneous tumor (I), and from Ehrlich carcinoma (II).

planted tissues grew actively. However, the epithelial production of normal tissues and of spontaneous tumors always ceased after 7 or 8 days' cultivation even if the cultures were washed regularly and given new nutritive fluid. But the epithelial migration of Ehrlich carcinoma continued for a much longer period of time. Measurements of the rate of epithelial expansion gave curves obviously different from those of Ehrlich carcinoma cultivated in the same flask (Text-fig. 1). Furthermore, explants from normal as well as from spontaneous tumor tissues diminished in size when the epithelium migrated. According to their size and the type of the tissue, they formed more or less rapidly a thin membrane consisting exclusively of a single layer of cells. They did not increase in volume while, under identical conditions, the fragments of Ehrlich carcinoma became larger. In such cultures, the epithelial membranes showed only a few cells in mitotic

division, again in obvious contrast to cultures derived from Ehrlich carcinoma tissue, which contained an abundance of dividing cells[9] (Figs. 4 and 5). The ameboid cells, at first active, soon degenerated and disappeared. The fibroblasts showed little activity and also degenerated in a short time. They never had any tendency to overgrow the epithelium.

Tissue Extracts.—Chicken, mouse, and rat embryonic extracts did not differ in their effects on mouse tissues. The optimal concentrations for producing epithelial migration were determined for chicken and mouse extracts. Both normal and tumor epithelium proved very sensitive to changes in concentrations. With concentrations rising to 20 per cent, epithelial activity increased. Migration was optimum when the concentrations reached between 10 and 20 per cent. It decreased when the concentration was over 25 per cent, and cell degeneration started more rapidly. These findings correspond to those of Fischer[10] for Ehrlich carcinoma, although the actual figures are slightly different, since Fischer found optimal growth at about 30 per cent, and rapid degeneration at over 40 per cent. Zakrzewski,[11] working with another transplantable mouse carcinoma, obtained optimal activity at about 6 per cent, and toxic effects in concentrations over 15 per cent. Extracts from adult mouse organs, either from a tumor-bearing or a normal animal, gave similar results. They were always found less satisfactory than embryonic extracts at similar concentrations. Extracts from both embryo and adult organs, even in the respective optimal concentrations, brought about epithelial migration far less readily than rat serum. On the other hand, they were more favorable than the latter to other cell types, such as fibroblasts and ameboid cells.

The duration of cell activity was limited. As far as could be observed without precise measurements, the activity of fibroblasts and ameboid cells was more marked and lasted longer in embryonic extracts than in rat serum. Nevertheless both cell types soon degenerated in all the media tested, even when the cultures were regularly treated and transferred. In no instance did they manifest greater or longer activity than the epithelium did.

Although, as stated before, epithelial tissues grew more rapidly in rat serum at a concentration of 50 per cent than in any other medium, this fluid did not allow continued migration of any tissue except of Ehrlich carcinoma. Even if the transfers to new medium were technically successful, the onset of migration was delayed. The rate of migration, furthermore, diminished at every subsequent passage and finally ceased entirely, regardless of whether the cultures were placed on the surface of the coagulum or inside it. The duration of cell activity varied according to the nature of the tissues. Normal tissues, such as epithelium from the mammary gland and from spontaneous tumors, invariably failed to show any

[9] Fischer, A., and Parker, R. C., *Brit. J. Exp. Path.*, 1929, 10, 312.

[10] Fischer, A., *Z. Krebsforsch.*, 1928, 26, 228, 239.

[11] Zakrzewski, Z., *Z. Krebsforsch.*, 1930, 30, 106.

activity after 3 or 4 weeks. Epithelium from Ehrlich carcinoma, on the other hand, was still active after 5 months. Fischer has been able to cultivate such epithelium for over 4 years.[12] Rat serum, although the best nutritive medium for all the tissues studied, supported the growth of normal or spontaneous tumor epithelium for a limited period only.

Fragments of Normal Tissues.—Fischer[1,12,13] found that fragments of Ehrlich carcinoma placed next to normal tissue used it as an artificial stroma. They fed upon the normal cells. A number of similar experiments have been undertaken. Fragments of mouse, rat, and chick embryos, and of lung, liver, spleen, kidney, heart, testicle, placenta, and ovary of adult mice, either fresh or kept in the ice box, were placed in contact with tumor tissues. Both the hanging drop and the flask methods were used, with mouse and chick embryonic extracts and rat serum as nutritive media. The findings varied slightly, according to the type of tumor cultivated. The behavior of Ehrlich mouse carcinoma and of spontaneous tumors differed strikingly (Figs. 6–8). The former readily invaded the normal tissue in thick bud-like formations. The latter either pushed away the normal cells, which remained sharply distinct from them, or formed delicate tubules, which grew slowly and were incapable of actively invading the normal tissues. If subcultured, the Ehrlich carcinoma overgrew the normal tissues and could again be obtained in pure strain.[1,12,13] The spontaneous tumor, on the contrary, remained clearly defined, surrounded by the normal tissue. The duration of its activity was no greater. The cultures of Ehrlich epithelium could be divided repeatedly and their number considerably increased. This was not possible in the case of spontaneous tumors. Similar results were obtained when normal tissues were fresh, degenerated, or dead.

DISCUSSION

The aptitude of normal and malignant cells of the mouse to liquefy fibrin is a serious obstacle to prolonged cultivation *in vitro*. According to Carrel and Ebeling,[14,15] malignant cells generally do not attack

[12] Fischer, A., Gewebezüchtung: Handbuch der Biologie der Gewebezellen *in Vitro*, Munich, Rudolph Müller and Steinicke, 3rd edition, 1930.

[13] Fischer, A., *Compt. rend. Soc. biol.*, 1927, 96, 1118.

[14] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1928, 48, 105.

[15] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1928, 48, 285.

heterologous fibrin. With mouse tissues, the most satisfactory results were obtained with solid media composed of diluted chicken plasma alone. Such clots, if thoroughly washed, do not inhibit the migration of epithelial cells, and are much less readily liquefied than those containing mouse or rat plasma.

The main difficulty to overcome in the cultivation of mouse epitheliums is not to find the right solid medium, but to discover the appropriate nutritive environment. All epithelial cell types, both from normal organs and tumors, manifested their optimum activity in media containing rat serum. On the other hand, mouse tissues were found to degenerate far more quickly in mouse than in rat serum. Chicken serum appeared also to inhibit epithelial activity. Furthermore, extracts from embryos and adult organs proved less stimulating than rat serum, although slightly more favorable than the latter to the migration of connective tissue and blood cells. In Tyrode solution alone, the tissues did not manifest any activity. It was observed that cell migration, except in the case of epithelium from Ehrlich carcinoma, rapidly decreased and finally ceased entirely in all media tested, namely, in media containing embryonic extracts and sera of various origin, (mouse, rat, and chicken), either with or without the addition of living or dead normal tissues. Therefore, it seems obvious that these fluids lack the necessary substances for prolonged growth *in vitro* of the epithelial cells, or that these cells, under the conditions of the experiments, are not able to utilize the substances contained in their medium. Besides, it is possible, as suggested by Zakrzewski,[11] that the explants are inhibited by their own metabolic products, even if regularly washed.

These negative results indicate that the nutritive media that determine the unlimited growth of Ehrlich mouse carcinoma and of normal as well as malignant tissues from animals of other species are not suitable for the cultivation of adult mouse epithelium derived from normal organs or from spontaneous mammary gland tumors. The prolonged cultivation of pure strains of mouse epitheliums will probably be unsuccessful until the discovery of new technical procedures or culture media differing from those hitherto used.

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tures of chicken epithelium are easily contaminated by connective tissue cells and overgrown by them. Mouse epithelium·showed, on the contrary, greater activity than connective tissue cells from the same origin and overgrew them. Especially in media containing rat serum, the fibroblasts disappeared spontaneously from mixed cultures. This property of mouse epithelium renders unnecessary the special techniques required in the cultivation of chicken epithelium,[7] as pure strains are spontaneously obtained. Adult mouse epithelium, derived from either normal organs or from spontaneous mammary gland tumors, could not be kept alive *in vitro* over prolonged periods of time. It grew better in rat serum than in embryonic juices. Both fibroblasts and iris epithelium from chicken, on the other hand, can be kept proliferating *in vitro* for a considerable period of time with embryonic juice as nutritive medium, but they do not thrive in rat serum. Tissues from other animals, as *e.g.*, dog, cat, rabbit, guinea pig, and rat, according to Carrel,[16] show similar nutritional requirements *in vitro* to chicken tissues. It is obvious that mouse tissues possess some very peculiar nutritive properties. Earlier investigators, especially Drew,[17] Fischer,[12] and Zakrzewski,[11] also agree that normal adult mouse tissues are most difficult to cultivate. This explains why no accurate comparison has been made so far of the normal and tumor tissues of the mouse, although the properties of some malignant cells from experimental mouse tumors, namely epithelium from Ehrlich mouse carcinoma[1,12] and another transplantable carcinoma and one sarcoma[11] have been the subjects of extensive studies.

On account of the peculiarities exhibited by mouse epitheliums, it is useless to compare the nutritional requirements of Ehrlich carcinoma, so thoroughly described by Fischer,[1,10,12] with those of normal epithelium from other animals. The malignant epithelium of the mammary gland of the mouse must be compared with the normal mammary gland epithelium of the same animal. Both normal tissues and spontaneous tumors are almost identical in their nutritional requirements. In fact, it was impossible to detect any

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[16] Carrel, A., *Compt. rend. Soc. biol.*, 1927, 96, 603.

[17] Drew, A. H., *Brit. J. Exp. Path.*, 1923, 4, 46.

difference between them under the conditions of the experiments. Their properties, however, differ from those of the Ehrlich carcinoma. In media containing rat serum or embryonic extracts, which latter determined the indefinite growth of Ehrlich carcinoma, spontaneous tumors grew for a limited period only. The best growth *in vitro* of the normal mammary gland and of spontaneous tumors of the same organ was observed in rat serum. Another difference between Ehrlich carcinoma and other epithelial tumors was the reaction of the epithelium toward normal tissues. The procedure of Fischer,[1,12,13] who placed such tissues in the medium as an artificial stroma for the tumor cells, succeeded only in the case of Ehrlich carcinoma. The hope that such a procedure could be used for the isolation of the epithelial constituents of any tumor has not been fulfilled. Laser,[18] while studying the transplantable Flexner-Jobling carcinoma, found that the cells of this tumor invade normal tissues *in vitro* with difficulty. In a recent investigation by Zakrzewski[11] of three other transplantable tumors (Jensen sarcoma, one mouse carcinoma, and one mouse sarcoma), no invasion of the normal tissues occurred. Negative results were likewise obtained in all spontaneous epithelial tumors of the mammary gland that were tested in this laboratory. The tumor cells behaved like normal chicken epithelium when cultivated with fibroblasts of the same animal. They did not infiltrate normal tissues, but remained separated from them by a well defined line.

Still another difference was observed between the epitheliums of Ehrlich carcinoma and of spontaneous tumors. Although both cell types attacked fibrin from rat and mouse plasma more readily than fibrin from chicken plasma, the epithelium from spontaneous tumors exhibited a greater capacity to liquefy the coagulum than the epithelium from Ehrlich carcinoma.

During the course of the experiments, other peculiarities were brought to light in the mode, rate, and duration of epithelial expansion of spontaneous tumors, in their proteolytic manifestations, in their invasive property toward normal cells, and in their production of acid. In a subsequent article, these variations will be reported in greater detail and an attempt be made to correlate these differences with the microscopical structure and the degree of malignancy of the tumors.

[18] Laser, H., *Z. Krebsforsch.*, 1927, **25**, 297.

CONCLUSIONS

The cultivation *in vitro* of mouse tissues derived from normal organs from 86 spontaneous epithelial tumors of the mammary gland, and from 27 Ehrlich carcinomas, has been undertaken, together with a study of the properties of the various cell types.

1. The tissues liquefied fibrin from mouse and rat plasma more readily than fibrin from chicken plasma. Clots made of chicken plasma alone, if thoroughly washed, did not inhibit the migration of the cells. Normal and tumor tissues liquefied fibrin from the mouse, rat, and chicken more actively than Ehrlich carcinoma did.

2. Mouse epitheliums, both normal and malignant, showed greater activity than connective tissue cells from the same origin and were not overgrown by the latter.

3. Mouse epithelium was more active in rat serum than in mouse or chicken serum and in embryonic juice from chickens, mice, and rats. None of these fluids, however, supported cell proliferation indefinitely except in the case of Ehrlich carcinoma.

4. These results indicate that mouse tissues possess nutritional requirements which are different from those of fibroblasts and epithelial cells of other animals. Nutritive media that suffice for prolonged cultivation of the normal and malignant tissues of the rat and the fowl, and also of Ehrlich carcinoma, are not suitable for the cultivation of adult mouse epithelium derived from normal organs or from spontaneous mammary gland tumors.

5. Rat serum supported the life of spontaneous tumors for a limited period of time only, whereas it enabled the Ehrlich carcinoma to proliferate indefinitely. Normal organs and spontaneous tumors were not capable of invading normal tissues as Ehrlich carcinoma did.

EXPLANATION OF PLATES

PLATE 14

FIG. 1. Hanging drop culture of normal mammary gland from a pregnant mouse. After 6 days' incubation, fixed and stained with hematoxylin and eosin. × 54.

FIG. 2. Flask culture of spontaneous mammary gland tumor from a mouse, after 4 days' incubation in 50 per cent rat serum as nutritive medium. × 38.

FIG. 3. Same flask as in Fig. 2. Culture of Ehrlich carcinoma, after 4 days' incubation in 50 per cent rat serum as nutritive medium. × 38.

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PLATE 15

FIG. 4. Hanging drop culture of normal mammary gland. After 6 days' incubation, fixed and stained with hematoxylin and eosin. × 480.

FIG. 5. Flask culture of Ehrlich carcinoma. After 16 days' incubation, fixed and stained with hematoxylin. Second passage. × 480.

PLATE 16

FIG. 6. Flask culture of spontaneous mammary gland tumor, and embryonic fibroblasts. Fixed and stained with hematoxylin and eosin. × 53.

FIG. 7. Flask culture of spontaneous mammary gland tumor from a mouse, and embryonic fibroblasts. Fixed and stained with hematoxylin and eosin. No invasion of normal tissue by tumor cells. × 37.

FIG. 8. Ehrlich mouse carcinoma and embryonic fibroblasts. Fixed and stained with hematoxylin and eosin. Normal tissue invaded by carcinomatous epithelium. × 101.

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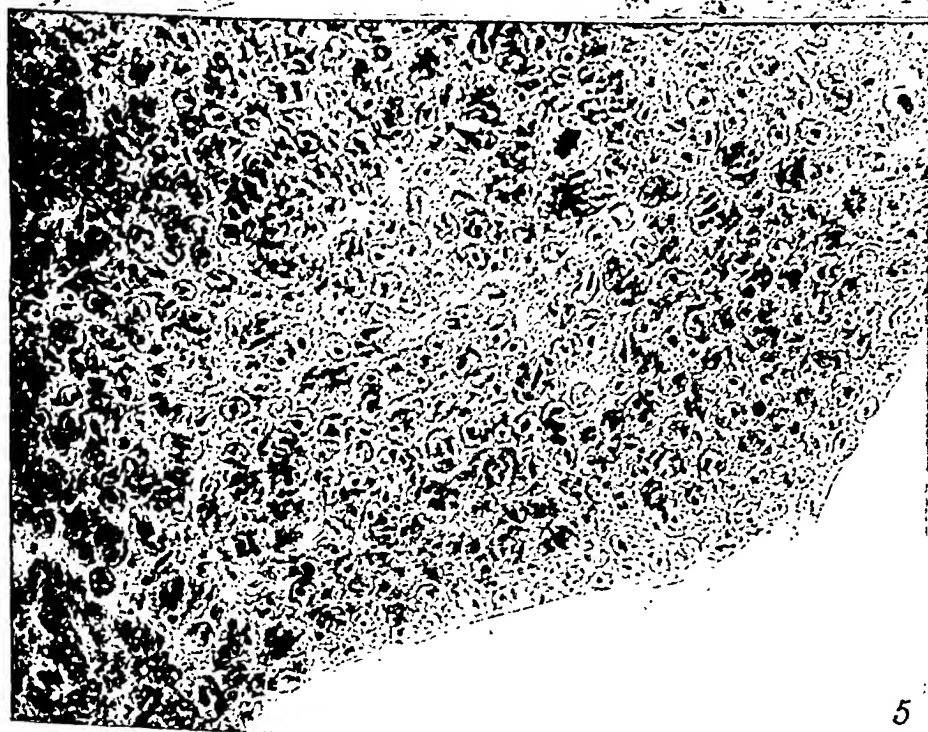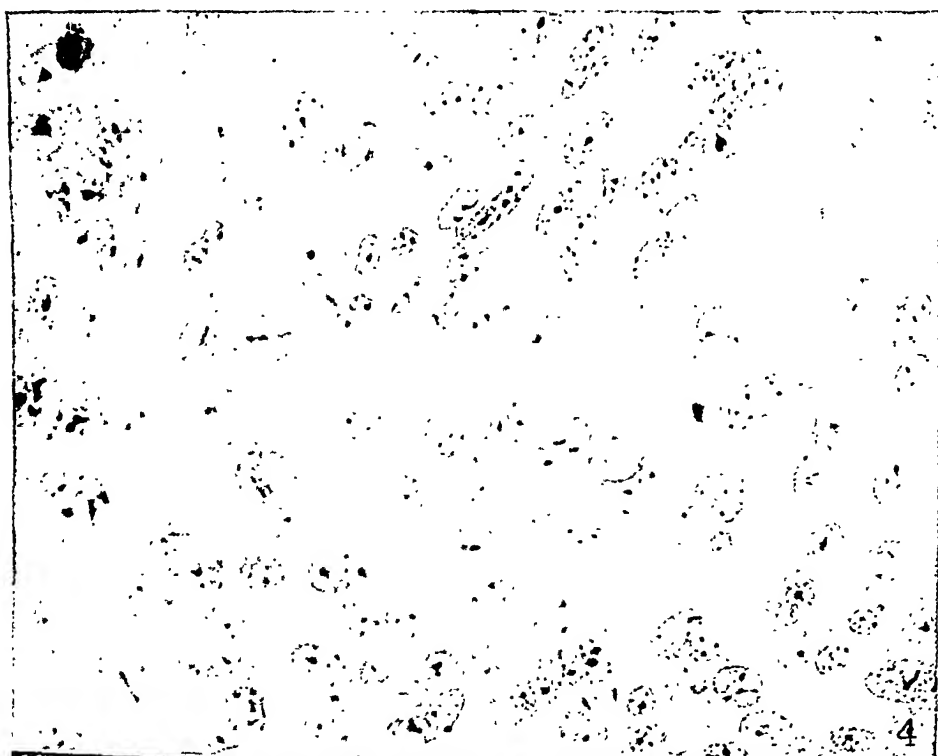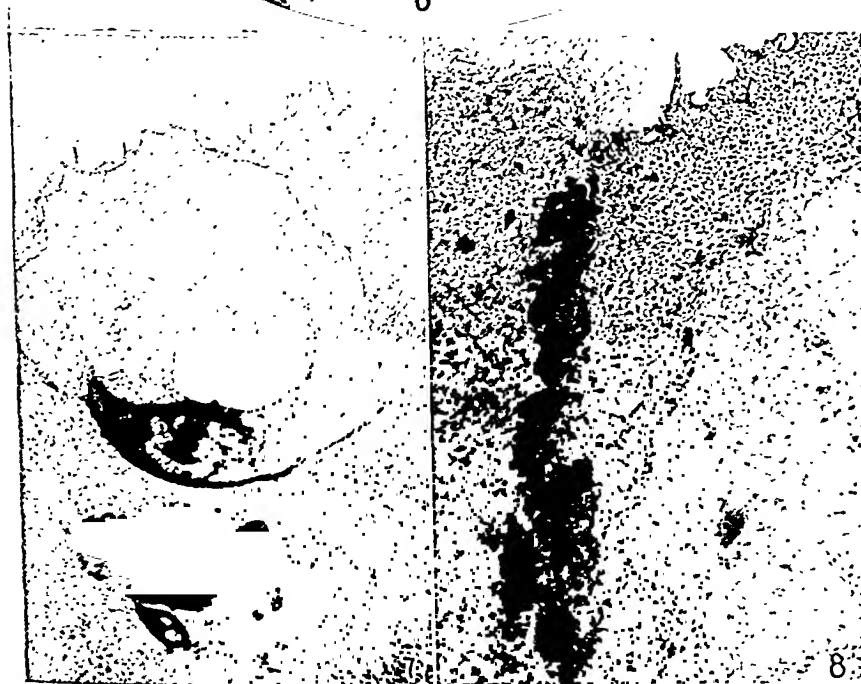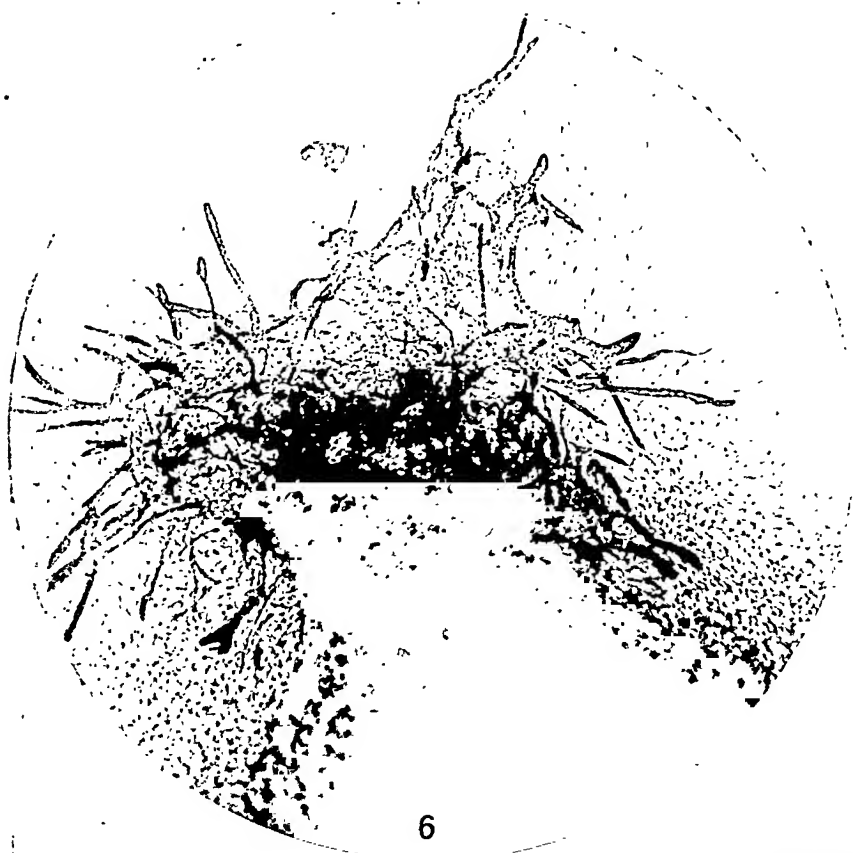
EXPERIMENTAL NEPHRITIS IN THE FROG

IV. THE SIGNIFICANCE OF THE FUNCTIONAL RESPONSE TO VASCULAR AND TO PARENCHYMAL DISTURBANCES IN THE KIDNEY*

BY JEAN OLIVER, M.D.

(From the Department of Pathology of the Long Island College of Medicine, The Hoagland Laboratory, Brooklyn)

PLATES 17 AND 18

The fundamental problem which in the last analysis confronts both the clinical and experimental investigator of the kidney is the deduction of the future state of the organ's activity from its present status. It is thus only a special case of the general principle of scientific prediction of future phenomena from present observable conditions.

The two methods which have been evolved to meet this problem may be summarized as the anatomical and the functional approaches. Whereas the first of these deduces from the observation of structural alterations the probable course of the organ's future activity, the latter by an immediate examination of function measures activity directly and claims therefore to speak more authoritatively. The dictum that "it is more important to know what the kidney is doing than what it looks like" is an aphoristic summary of this attitude, a statement which at its face value would seem to be almost self-evident, until one is confronted by the confusion that has followed when the results of its application have been compared with those of the anatomical method. Such attempts at correlation of structure and function must of necessity be made since they are the basis of modern medicine, but before proceeding to the investigation by either method of multiple and highly complex details it would seem wise to examine this fundamental problem in as exact a manner as possible. Though it will be manifestly impossible for one limited group of observations to more

high titre to be useful in laboratory tests, without adding large quantities of normal serum to the broth. Experiments recorded in this paper show that serum affects hemolysin in unexpected ways, causing delayed hemolysis, resistance to oxidation or reduction, and inability to combine with the specific antihemolysin which readily neutralises yeast extract streptolysin. An experiment has been quoted showing that although an immune serum was ten times more active than a normal serum in neutralising yeast extract hemolysin, yet these two sera were equal in their ability to neutralise serum hemolysin. It therefore seems probable that the neutralisation of serum streptolysin by normal and immune sera is dependent on the chemical composition of the sera and is not due to the action of a specific antibody. This hypothesis is supported by the observations of Lyall (6) who, working with guinea pig, rabbit, cow, human, and sheep sera showed that, with occasional variations, the volume of serum required to neutralise a unit of serum hemolysin is uniform for the same species of animal. McLeod and McNee (7) showed that rabbits become anemic when injected with serum streptolysin and that repeated injections not only fail to cause immunity but actually render relatively insusceptible rabbits more susceptible to serum hemolysin. This observation is in marked contrast to the experiments on immunity to yeast extract streptolysin recorded in this paper which show that repeated injections of active yeast extract streptolysin produce only transitory toxic symptoms in rabbits and stimulate the production of specific antihemolysin. It is also possible to demonstrate that animals immunised with yeast extract streptolysin are more resistant to infection by the relatively attenuated variants of hemolytic streptococci than normal animals, although their immunity to highly virulent variants is not increased. These important differences between the properties of two substances which possess certain common characteristics, but which are obviously not identical, necessitate consideration of the relationship which presumably exists between serum hemolysin and yeast extract hemolysin. It is possible that serum streptolysin owes its distinctive characteristics, namely filterability, relatively slow lysis, resistance to oxidation or reduction, and absence of antigenicity, to adsorption by serum; and that the free streptolysin which is formed in cultures without serum owes its greater lability and activity to the absence of any adsorbing substance.

In this experiment the complicating conditions which had prevented a just comparison in the previous experiments were ruled out. The epidemic of pneumonia attested to the fact that the controls were exposed to intercurrent infection. It carried off principally the ill-nourished mice, those, that is to say, in which cancer was less likely to develop. Nevertheless the incidence of cancer was practically identical in the two groups (Charts 6 and 7). What the charts fail to show is that on the average the warts and cancers of the sheltered and control mice differed not at all in character, rate of growth, and tendency to be multiple.

<center>COMMENT</center>

The fact emerged from our first experiments that sheltering adult mice from the access of living entities by way of food, drink, bedding, and air was insufficient to prevent the development of cancer in a considerable proportion of them, when the appropriate stimulus of tissue injury was provided. It remained for Experiment 3 to show, however, that the incidence of cancer was absolutely unaffected by this precaution. Nor was it influenced in the least by the profound difference in the character of the food given the controls and the sheltered animals. Tarred mice fed exclusively upon raw mammalian muscle became cancerous with precisely the same frequency as those subsisting largely upon cereals. The finding accords with that of Leonard Hill who placed five groups of mice on differing diets and could detect no significant influence of these on the incidence of tar cancer (6).

The periods of 118 to 196 days during which our animals were sheltered comprise from one-sixth to more than one-fourth of the span of life of the mouse, assuming this to be 2 years, a generous estimate. In Experiment 3 the sheltering had lasted for more than 5 weeks before any injury was produced of the sort that ultimately led to cancer. The results prove that mouse cancer cannot be caused by living entities reaching the body from the surrounding world during adult life, and they rule out for this species the possibility of the prevention of the disease by methods based on such an assumption. They fail to exclude the possible activity of entities residing habitually in or upon the body, as do the staphylococci and the colon bacillus.

In the second experiment the vascular disturbance was produced in the circulation of Segment II of the tubules by restricting the flow through the renal-portal system. Chart 2 A shows the alterations in urine formation that developed.

In the first two 15 minute periods the rates of excretion of water, salts and neutral red averaged 8.0 cc. and 20.0 and 2.2 mg. per hour, and the urine was free of

Glomerular Dysfunction

CHART 1

sugar. At the end of the second period the flow to the tubules through the renal-portal system was reduced to one-tenth its original value. A sharp fall in the excretion of neutral red occurred which finally reached the rate of 0.8 mg. per hour. The excretion of water and salts increased on the other hand to rates of 18.0 and 118.0 mg. per hour and sugar appeared in the urine.

2. *The Results of a Functional Testing of Kidneys in Which There Are Parenchymal Disturbances.*—For the production of alterations in the

JEAN OLIVER

parenchyma of the kidney a procedure was used the action of which has been studied both clinically and experimentally. Corrosive sublimate produces marked disturbances in both the epithelial elements, particularly in Segment II of the tubules, and in the glomeruli. Furthermore we have previously demonstrated that all of the essential lesions that develop *in vivo* may be produced extravitally when the toxic substance is introduced into the perfusion circulation of the iso-

Tubular Dysfunction
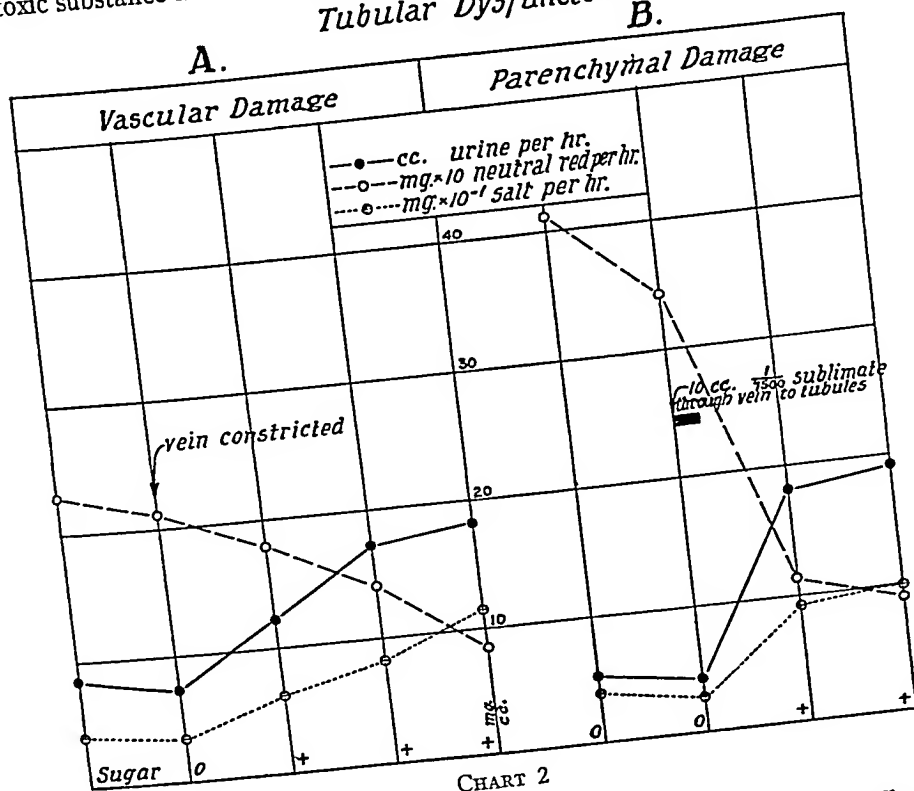
CHART 2

lated kidney (3). The following typical experiment shows the effect of such a procedure directed towards the glomerular apparatus as tested by functional examination. The results are summarized in Chart 1 B.

In the first period of normal perfusion the rates of excretion of water, salts, urea and phenol red were 12.0 cc. and 90.0, 9.0 and 1.2 mg. per hour. During the

that hemolysin, inactivated by exposure to air at 38°C. for 6 hours, could be reactivated by reducing substances such as sodium hydrosulfite, but that more prolonged incubation caused irreversible oxidation. They therefore distinguished three degrees of activity of streptolysin: (1) active hemolysin in the fully reduced state, (2) inactive hemolysin which could be reactivated by reduction, (3) inactive hemolysin which was irreversibly oxidised and which could not be reactivated by reducing substances.

Todd (4) showed that streptolysin, obtained from broth cultures containing 20 per cent of normal horse serum, did not react with oxygen in the manner described by Neill and Mallory. The titre of hemolysin in serum broth gradually fell on exposure to incubator temperature and it was found impossible to prevent this decline in activity by exclusion of oxygen. It was also found that inactivation was not accelerated by powerful oxidising agents such as hydrogen peroxide.

The following experiments were done to investigate these discrepancies.

Hemolytic streptococci were grown in broth to which 20 per cent of normal horse serum had been added; and sterile hemolysin was obtained by filtration through a Maasen candle. The hemolysin was titrated immediately after filtration and it was then completely inactivated by storage in a 5 x ¾ inch tube at 25°C. for 2 days. A parallel experiment was performed with a culture of the same strain which had been grown for 16 hours in broth containing 7 per cent of yeast extract instead of serum. In this case inactivation at 25°C. was continued for an extra day to allow more complete oxidation. The two inactive filtrates, which gave the same pH readings, were then reduced with 1.0 per cent of sodium hydrosulfite and phosphate buffer as described by Neill and Mallory (30 minutes at room temperature). Table I shows that the serum-free hemolysin was reactivated to a higher titre than the original filtrate (Ly_r + Ly_o Neill and Mallory); on the other hand sodium hydrosulfite did not cause any return of hemolytic activity in the filtrate which contained serum.

Channon and McLeod (5), working with broth containing 30 per cent of normal ox serum, succeeded in obtaining, by reduction, a slight reactivation of streptolysin which had been inactivated at 37°C. They noted, however, that only a partial reactivation was obtained (M.H.D. of original filtrate = 0.01 cc., M.H.D. of reduced filtrate = 0.5 cc.) in contrast to the experiments of Neill and Mallory who obtained,

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sults of vascular and parenchymal damage. This was true, as is emphasized in the arrangement of Charts 1 and 2, in the case of both glomerular and tubular dysfunction for it is seen that the type of functional derangement is identical in the two types of damage. Anatomical examination of the kidneys on the other hand showed definite differences in the state of the kidneys in the two types of damage, whether the dysfunction was glomerular or tubular.

Certain points should be emphasized here. First, the validity of these results is not dependent on any particular interpretation of the significance of the functional phenomena observed. Whatever the anatomical relations between the two circulations in the kidney, whether urea, salts, dyes or water is excreted by one mechanism or another, no matter what part "filtration" or "absorption" may play in the elaboration of the final urine, the fact remains that the status of the function of these kidneys was identical, no matter how its functional state came into being, when an anatomical examination showed their actual condition to be significantly different.

The fact that vascular disturbances, if of sufficient duration may in turn produce parenchymal changes complicates the problem still further, for in lesions that spontaneously develop in the kidney the mixture of vascular and parenchymal disturbances is so intimate that the functional results become infinitely more difficult of interpretation. Our previous studies have shown that even in the controlled extravital experiment conditions and relations of functional and structural response may thus become exceedingly complex (2). These complications were purposely avoided in the present study, however, by making the period of vascular disturbance short. Also, and again for the purpose of simplification, the toxic agent which caused the parenchymal disturbances was used in low enough concentration to produce only the less complex of the structural alterations that may follow its contact with the cells. And for the same reasons the simplicity of the general conditions existing in the perfusion experiments deserves special emphasis. Every element of the circulating fluid that is going to the kidneys is known and may be varied at will. Every constituent of the urine formed from this fluid can be accurately determined and compared with its condition in the circulating fluid. We have given in our experiments only rates of excretion but "con-

centration factors," "ratios," "clearances" or any other formulae might be calculated, without altering the conclusion that the functional status of the organs in the two types of damage, vascular and parenchymal, was identical.

All these contrasts between the simplicity of our experiments and the complexity that must obtain when the problem is investigated in the living animal, particularly if mammals are used whose renal activity is only partially understood, add considerable weight to the conclusion that functional examination is unable to differentiate between two types of damage of very different significance, the one vascular, transient and reversible, the other parenchymal, permanent and, as far as the cells involved are concerned, irreparable.

It might seem that a similar result is the proper conclusion to be drawn from the long series of similar attempts by clinical and experimental study to determine the condition of the kidneys from functional examinations. But it has been and apparently still is hoped, perhaps because in such examinations relations are so complex and involved that nothing seems beyond hope, that some refinement in method or the use of some selectively excreted substance, such as a dye or other foreign substance, may distinguish between the two conditions of vascular and parenchymal disturbance. The answer of our experiments is that the apparent *similarity* in the findings of the functional tests in the two cases is in fact an *identity* in the functional state in the two conditions though produced by different mechanisms; and there remains no reason to suppose that any procedure could distinguish between differences that in fact do not exist.

The observation of the anatomical changes in the kidneys of our experiments allowed on the other hand a ready determination of the significance of the alteration that existed in the two conditions of damage, since the fate of the organ could be predicted directly from the structural alterations observed. In our experiments the observations were made by histological means; other methods which have been shown by postmortem pathological evidence to be valid and to give similar information, are however available (7).

A final point in these experiments may be emphasized, well known perhaps, but often insufficiently appreciated, namely, a weakness in the anatomical approach to the problem. The morphologist is un-

CONCLUSIONS

The cultivation *in vitro* of mouse tissues derived from normal organs from 86 spontaneous epithelial tumors of the mammary gland, and from 27 Ehrlich carcinomas, has been undertaken, together with a study of the properties of the various cell types.

1. The tissues liquefied fibrin from mouse and rat plasma more readily than fibrin from chicken plasma. Clots made of chicken plasma alone, if thoroughly washed, did not inhibit the migration of the cells. Normal and tumor tissues liquefied fibrin from the mouse, rat, and chicken more actively than Ehrlich carcinoma did.

2. Mouse epitheliums, both normal and malignant, showed greater activity than connective tissue cells from the same origin and were not overgrown by the latter.

3. Mouse epithelium was more active in rat serum than in mouse or chicken serum and in embryonic juice from chickens, mice, and rats. None of these fluids, however, supported cell proliferation indefinitely except in the case of Ehrlich carcinoma.

4. These results indicate that mouse tissues possess nutritional requirements which are different from those of fibroblasts and epithelial cells of other animals. Nutritive media that suffice for prolonged cultivation of the normal and malignant tissues of the rat and the fowl, and also of Ehrlich carcinoma, are not suitable for the cultivation of adult mouse epithelium derived from normal organs or from spontaneous mammary gland tumors.

5. Rat serum supported the life of spontaneous tumors for a limited period of time only, whereas it enabled the Ehrlich carcinoma to proliferate indefinitely. Normal organs and spontaneous tumors were not capable of invading normal tissues as Ehrlich carcinoma did.

EXPLANATION OF PLATES

PLATE 14

FIG. 1. Hanging drop culture of normal mammary gland from a pregnant mouse. After 6 days' incubation, fixed and stained with hematoxylin and eosin. × 54.

FIG. 2. Flask culture of spontaneous mammary gland tumor from a mouse, after 4 days' incubation in 50 per cent rat serum as nutritive medium. × 38.

FIG. 3. Same flask as in Fig. 2. Culture of Ehrlich carcinoma, after 4 days' incubation in 50 per cent rat serum as nutritive medium. × 38.

ON THE NUTRITIONAL REQUIREMENTS IN VITRO OF NORMAL AND MALIGNANT MOUSE EPITHELIUMS

By LARS SANTESSON

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

PLATES 14 TO 16

In order to gain a better knowledge of the fundamental properties of normal and malignant tissues, it is desirable to study *in vitro* various cell types obtained from normal tissues and from experimental and spontaneous tumors. In this manner, normal and malignant tissues can be accurately compared. Such a comparison must be made on pure cultures and between cells from the same animal species. Up to now, it has not been possible to compare the only malignant epithelium of the mouse yet obtained in pure culture, that is, the epithelium from Ehrlich carcinoma,[1] with less malignant or with normal homologous epitheliums, since the techniques necessary for obtaining pure strains of mouse tissues have still to be discovered. The purpose of the present paper is to describe some relations between the nutritional requirements *in vitro* of epithelium derived from normal organs of adult mice and those of epithelium from spontaneous and transplanted mammary gland tumors.

EXPERIMENTAL

Normal Tissues.—The mammary gland is embedded in fat and composed of only a few epithelial tubules. Therefore, the probability of cutting through epithelial tissue, when preparing fragments for cultivation, is small. Even in suitable media, epithelial migration does not regularly occur. It is necessary to make a considerable number of explants from each gland in order to obtain a few results. Epithelial migration occurred most frequently in cultures of glands of animals in early pregnancy, whereas epithelial cells from glands of

[1] Fischer, A., *Z. Krebsforsch.*, 1927, 25, 89.

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(Oliver: Experimental nephritis in the frog. IV)

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THE MULTIPLICATION OF THE VIRUS OF MEXICAN TYPHUS FEVER IN FLEAS

By H. MOOSER, M.D., and M. RUIZ CASTANEDA, M.D.

(From The American Hospital, Mexico City, Mexico, and the Laboratory of the Department of Bacteriology and Immunology, Harvard University Medical School, Boston, Massachusetts)

PLATE 19

In two preceding publications, (1, 2) the writers, with Zinsser, have reported the discovery of the virus of Mexican typhus fever in rats trapped in Mexico City, and have described the conditions under which the virus can be conveyed from rat to rat by the rat louse, *Polyplax spinulosus*. These investigations brought definite proof that there is an animal reservoir of typhus virus outside the human body from which epidemic spread can occur under suitable conditions. The appearance of epidemics after prolonged typhus-free intervals is far more readily explained by the existence of such an animal reservoir than by the older hypothesis of a continuous maintenance of the typhus virus in an apparently free community by the occurrence of inapparent human infections. It is hardly conceivable that, in the prolonged interepidemic periods, all or even the majority of human cases which bridge the interval between two epidemic outbreaks should remain clinically inapparent.

In order to discover the means of transmission of the disease from rat to man, it became necessary to study in detail the blood-sucking arthropods which infest the living rats in Mexico City. An investigation of several months in which insects were collected from trapped rats revealed the following ectoparasites.

Mites: *Laelaps echidninus*
Liponyssus bacoti[1]

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[1] We are indebted to Dr. Ewing of the U. S. Department of Agriculture for the identification of this mite.

Lice: *Polyplax spinulosus*
Fleas: *Xenopsylla cheopis*
Leptopsylla musculi
Ceratophyllus fasciatus
Ctenocephalus canis (rare)
Ctenocephalus felis (rare)

Of the mites, *Laelaps echidninus* was shown to be unable to transmit the disease, as no virus could be found in this species in spite of prolonged contact with infected rats. This mite does not ingest blood during its larval and nymphal stages, and of the adults only the female has been reported to suck blood.

Liponyssus bacoti, on the contrary, takes blood during its larval, nymphal and adult instars. When put on rats, it becomes engorged with blood in less than 48 hours, as a rule, whereupon the nymphs and adult stages drop off and crawl to a hiding place, where they moult or lay eggs according to their respective developmental stages. The larvae may remain several days on the host before they crawl off. Within less than a week most of the mites are ready for another meal. In two experiments, in each of which we used numerous mites and examined them between 9 and 10 days after an infectious meal, we were not able to observe *Rickettsiae* or to demonstrate the virus of typhus in this species.

There seem to remain, therefore, only the fleas, which had already been shown by Dyer, Rumreich and Badger (3) to be able to harbor the virus of endemic typhus in the southern United States. Of the fleas found on our rats, all except *Leptopsylla musculi* have been reported to attack man more or less readily. We have, therefore, tested all of the above mentioned species systematically, in order to find out in which of them the virus of Mexican typhus is able to survive and to multiply. At the same time, we tried to determine which of these fleas would be most likely to be implicated as a vector from rat to rat and from rat to man. Our methods varied somewhat according to the species of flea which was to be investigated.

When rat fleas were tested (*Xenopsylla, Ceratophyllus, Leptopsylla*) they were, as a rule, put on the rats some time before infecting the latter with a heavy dose of guinea pig tunica emulsion. In the experiments with cat and dog fleas (*Ctenocephalus*) the starved insects were put on the rats immediately after the febrile

period had made its appearance, in view of the difficulty of keeping these two species on rats for any length of time. The rats were kept in high glass jars covered with fine mesh gauze. The average room temperature was around 20°C. The temperature in the jars oscillated between 26° and 28°C. during the daytime. In all these experiments, with the exception of those carried out by Dr. Castaneda with dog fleas, a typhus strain isolated from wild rats in Mexico City was used.

Experiments with Leptopsylla musculi.—

Large numbers of this species were found on two wild rats (*Mus rattus rattus*) caught in the vicinity of Mexico City. The rats were killed and put in a jar con-

Protocol 1
Guinea Pig 1

Date	Temperature	Remarks
July 14	°C.	
" 15	39.2	
" 16	39.3	Inoculated
" 17	39.2	
" 18	40.2	
" 19	39.6	
" 20	40.5	
" 21	40.8	Scrotum red and swollen
" 22	40.6	
" 23	39.7	
" 24	39.7	
" 25	39.6	
" 26	39.0	
" 27	39.1	
	39.0	

This animal was preserved for immunity test with Nicolle's strain of African typhus.

aining three young white rats. 24 hours later, on June 28th, the dead rats were emoved and the three white rats, Nos. 1, 2 and 3, inoculated with tunica emul-on. On July 7th, one flea was removed, smeared and stained with Giemsa solu-n. Numerous small red staining organisms were observed in this smear. They re indistinguishable from *Rickettsia prowazeki* as found in typhus-infected fleas. July 11th, large numbers of these organisms were observed in the smears of two er fleas. Three fleas were removed on that day and prepared for histological nination. On July 14th, two fleas were emulsified and inoculated into Guinea . The reaction of this animal is shown in Protocol 1. July 15th Rats 1, 2 and 3 were killed and a fresh rat, No. 4, put in the jar. the fleas had changed to the new rat, the others were removed. On July

20th, it was noticed that the number of fleas had decreased considerably on this rat. Five were recovered and smeared. Numerous typical *Rickettsiae* were found in all of them. On August 5th, Rat 4 was killed and its brain inoculated into a guinea pig. No symptoms resulted from this inoculation.

Experiments with Ceratophyllus fasciatus.—

Ten specimens of this species were procured from several wild rats (*Mus decumanus*) together with other fleas. The rats were killed and combed over a pan containing ice cold water. The fleas, anesthetized by the cold, were removed with a hair brush and classified. The collected specimens of *Ceratophyllus fasciatus* were then put on Rat 5, which several days later was inoculated with a tunica

Protocol 2

Guinea Pig 2

Date	Temperature	Remarks
	°C.	
Aug. 12		Inoculated with 3 fleas
" 13	38.7	
" 14	39.0	
" 15	40.0	Scrotum red and very swollen
" 16	39.5	
" 17	40.0	
" 18	40.2	Killed, autopsy typical. Tunica hemorrhagic, covered by a thick layer of fibrin. *Rickettsiae* present

Tunica emulsion inoculated into Guinea Pigs 3 and 4 produced typical typhus, with scrotum swelling within 4 days and numerous *Rickettsiae* in Guinea Pig 4. Guinea Pig 3 was preserved for immunity test with Nicolle's strain.

emulsion very rich in *Rickettsiae*. The results obtained with this species were in every respect identical with those of the preceding experiment. In smears of fleas examined around the 12th day after the inoculation of Rat 5 *Rickettsia prowazeki* was found in all of them, and the inoculation of an emulsion of three fleas into Guinea Pig 2 was followed by the typical picture of Mexican typhus. Two fleas were prepared for histological examination. The reaction of the guinea pig is shown in Protocol 2.

Experiments with Xenopsylla cheopis.—

This species was the most common flea found by us on rats in Mexico City during the spring and summer of 1931. It was therefore given special attention, its infectivity during a prolonged period after feeding on infected rats being thoroughly

investigated. Fleas collected in the harbor of Vera Cruz were used in one experiment, and fleas collected from rats in Mexico City in two additional experiments. 100 per cent of them were found to be infected with *Rickettsia prowazeki* when

Protocol 3
Guinea Pig 4

Date	Temperature	Remarks
July 9	°C.	
" 10	39.0	
" 11	39.0	
" 12	40.4	
" 13	40.3	Scrotum ++
" 14	40.2	" ++
" 15	40.3	" ++
" 16	40.2	" ++
" 17	40.0	" +
" 18	40.0	" +
" 19	39.7	" normal
" 20	39.5	" "
" 21	39.4	
" 22	39.0	
" 23	39.0	
	38.9	

Preserved for immunity test with Nicolle's strain.

Guinea Pig 5

Date	Temperature	Remarks
July 9	°C.	
" 10	38.9	
" 11	38.8	
" 12	40.2	Scrotum ++
	40.5	" +++. Killed. Numerous petechiae in the skin and subcutaneous tissue. Pronounced edema and hemorrhage around site of inoculation. *Rickettsiae:* numerous intra- and extracellular. Cultures negative

examined between the 9th and 10th days after the beginning of the febrile periods of the rats. The inoculation of emulsions of one and a half to two and a half fleas on the 13th,[2] 24th and 33rd days, respectively, into male guinea pigs was followed

[2] Counted from the day the rats had begun to show fever.

20th, it was noticed that the number of fleas had decreased considerably on this rat. Five were recovered and smeared. Numerous typical *Rickettsiae* were found in all of them. On August 5th, Rat 4 was killed and its brain inoculated into a guinea pig. No symptoms resulted from this inoculation.

Experiments with Ceratophyllus fasciatus.—

Ten specimens of this species were procured from several wild rats (*Mus decumanus*) together with other fleas. The rats were killed and combed over a pan containing ice cold water. The fleas, anesthetized by the cold, were removed with a hair brush and classified. The collected specimens of *Ceratophyllus fasciatus* were then put on Rat 5, which several days later was inoculated with a tunica

Protocol 2

Guinea Pig 2

Date	Temperature	Remarks
	°C.	
Aug. 12		Inoculated with 3 fleas
" 13	38.7	
" 14	39.0	
" 15	40.0	Scrotum red and very swollen
" 16	39.5	
" 17	40.0	
" 18	40.2	Killed, autopsy typical. Tunica hemorrhagic, covered by a thick layer of fibrin. *Rickettsiae* present

Tunica emulsion inoculated into Guinea Pigs 3 and 4 produced typical typhus, with scrotum swelling within 4 days and numerous *Rickettsiae* in Guinea Pig 4. Guinea Pig 3 was preserved for immunity test with Nicolle's strain.

emulsion very rich in *Rickettsiae*. The results obtained with this species were in every respect identical with those of the preceding experiment. In smears of fleas examined around the 12th day after the inoculation of Rat 5 *Rickettsia prowazeki* was found in all of them, and the inoculation of an emulsion of three fleas into Guinea Pig 2 was followed by the typical picture of Mexican typhus. Two fleas were prepared for histological examination. The reaction of the guinea pig is shown in Protocol 2.

Experiments with Xenopsylla cheopis.—

This species was the most common flea found by us on rats in Mexico City during the spring and summer of 1931. It was therefore given special attention, its infectivity during a prolonged period after feeding on infected rats being thoroughly

July 11. Weil-Felix of Rat 8 negative. One *Xenopsylla* prepared for histological examination.

Protocol 5

Guinea Pig 8

Date	Temperature	Remarks
	°C.	
Aug. 3		Inoculated
" 4	38.8	
" 5	39.0	
" 6	39.2	
" 7	40.2	
" 8	40.0	
" 9	40.4	
" 10	39.8	Scrotum +
" 11	40.1	
" 12	40.0	
" 13	39.6	
" 14	39.0	
" 15	39.0	
" 16	39.2	

Preserved for immunity test with Nicolle's strain.

Guinea Pig 9

Date	Temperature	Remarks
	°C.	
Aug. 3		Inoculated
" 4	39.1	
" 5	39.0	
" 6	39.4	
" 7	40.0	
" 8	40.6	Scrotum ++. Killed. Autopsy typical. Numerous endothelial cells heavily infected with *Rickettsiae*. Two successive passages in guinea pigs produced typical infections

July 13. Weil-Felix of Rat 8 negative.
July 14. Weil-Felix of Rat 8 positive 1–40.
July 15. Rat 8 found dead. Brain inoculated into a guinea pig which died from peritonitis on July 21st. Fresh rat, No. 9, put in cage.

in each instance by the typical picture of Mexican typhus after a very short incubation period, indicating an extraordinary multiplication of the virus.   Protocols 3 and 4 illustrate the reaction of two sets of guinea pigs inoculated with *Xenopsylla cheopis*.

Protocol 4
Guinea Pig 6

Date	Temperature	Remarks
	°C.	
July 28	·	Inoculated at 7 p.m.
" 29	39.3	
" 30	40.4	" " 10 a.m.
" 31	40.7	Scrotum +
Aug. 1	40.6	" ++. Killed. Autopsy typical. *Rickettsiae* in tunica ++++. Tunica into two guinea pigs which came down with typical picture of Mexican typhus

Guinea Pig 7

Date	Temperature	Remarks
	°C.	
July 28		Inoculated at 7 p.m.
" 29	39.3	
" 30	40.0	" " 10 a.m.
" 31	40.4	Scrotum ++
Aug. 1	40.7	" ++
" 2	40.6	" ++
" 3	40.3	" ++
" 4	40.0	" normal
" 5	39.3	
" 6	39.2	

Preserved for reinoculation with Nicolle's strain.

Experiment on Infection of Fleas on Inoculated Rats

June 23.   Two white rats previously infested with *Xenopsylla cheopis* of Mexico City inoculated with tunica emulsion.

June 25.   Both rats have fever.

June 29.   Both rats dead.   Replaced by healthy Rat 8.

July 6.   One flea examined.   *Rickettsia prowazeki* present.

July 8.   Three fleas emulsified and inoculated into Guinea Pigs 4 and 5—see Protocol 3).

July 11. Weil-Felix of Rat 8 negative. One *Xenopsylla* prepared for histo-logical examination.

Protocol 5

Guinea Pig 8

Date	Temperature	Remarks
	°C.	Inoculated
Aug. 3		
" 4	38.8	
" 5	39.0	
" 6	39.2	
" 7	40.2	
" 8	40.0	
" 9	40.4	Scrotum +
" 10	39.8	
" 11	40.1	
" 12	40.0	
" 13	39.6	
" 14	39.0	
" 15	39.0	
" 16	39.2	

Preserved for immunity test with Nicolle's strain.

Guinea Pig 9

Date	Temperature	Remarks
	°C.	
Aug. 3		Inoculated
" 4	39.1	
" 5	39.0	
" 6	39.4	
" 7	40.0	
" 8	40.6	Scrotum ++. Killed. Autopsy typical. Numerous endothelial cells heavily infected with *Rickettsiae*. Two successive passages in guinea pigs produced typical infections

July 13. Weil-Felix of Rat 8 negative.

July 14. Weil-Felix of Rat 8 positive 1–40.

July 15. Rat 8 found dead. Brain inoculated into a guinea pig which died from peritonitis on July 21st. Fresh rat, No. 9, put in cage.

July 25.   Rat 9 dead.   Replaced by fresh rat, No. 10.

July 28.   Eleven remaining fleas removed.   Five emulsified and inoculated into Guinea Pigs 6 and 7 (see Protocol 4).   Three fleas smeared and numerous *Rickettsiae* found.   Three fleas prepared for histological examination.

Experiments with Ctenocephalus felis.—

About 100 fleas collected from cats were put in a jar containing two white rats which 3 days previously had received a heavy dose of tunica emulsion.   3 days later only a few fleas could be observed on the rats, most of them having been killed.   8 days after the beginning of the experiment one flea was smeared and few, but typical, *Rickettsiae* found.   On the 10th day only a few fleas were still found on the rats.   One of them was prepared for histological examination.   The two remaining ones were emulsified and inoculated into Guinea Pigs 8 and 9, as indicated in Protocol 5.

Experiments with Dog Fleas.—

The fleas found on dogs in Mexico City are *Ctenocephalus canis*, *Ctenocephalus felis* and *Pulex irritans*.   In several experiments in which numerous dog fleas were put on infected rats, nearly all of them were destroyed by the hosts within a day or two.   These fleas bothered the rats greatly, and the rats did not spare any effort to get rid of them as quickly as possible.   Whereas rat fleas settle preferably on the heads of the rats, especially behind the ears and in the intermandibular groove, *Ctenocephalus* and *Pulex* settle on the abdomen, on the back and even on the legs, where they are reached easily by the teeth of the rats.   In several experiments, the few fleas which still remain on the rats after 8 to 9 days turned out to be nearly always *Ctenocephalus felis*.   *Rickettsiae* were found in them each time.   In one instance, however, a female *Ctenocephalus canis* was found on a rat on the 11th day after the beginning of the experiment.   It was emulsified and inoculated into Guinea Pig 10.

Experiment to Determine the Immunity to the Nicolle Strain of North African Typhus Fever of Guinea Pigs Inoculated with Infected Fleas

Guinea Pigs 1, 3, 4, 7, 8 and 10, all of them infected with flea material at various times, were used in this experiment.   The preliminary history and the date of inoculation as well as the materials inoculated into these guinea pigs are described in preceding experiments, where the corresponding numbers of these animals can be found.

On August 23, 1931, these six animals were inoculated with an emulsion of one-tenth of the brain of a guinea pig representing the 302nd passage of Nicolle's strain of North African typhus fever.   At the same time, four control guinea pigs were

inoculated with corresponding amounts of the same material. The amount of brain inoculated represented from 300 to 500 infectious doses for a guinea pig.

In order to economize space, we may summarize this experiment in the following way: None of the animals that had been flea-inoculated at a previous time and had developed typical Mexican typhus fever at that time showed a temperature reaction characteristic of the European or African typhus fever, though observed for 18 days. The highest temperature reached by any of them during this period was 39.3°C., or 102.74°F.

Protocol 6

Guinea Pig 10

Date	Temperature	Remarks
	°C.	
Aug. 1		
" 2		
" 3	39.4	Inoculated
" 4	39.2	
" 5	39.4	
" 6	39.6	
" 7	40.4	
" 8	40.6	
" 9	39.8	Scrotum +
" 10	40.2	" normal
" 11	40.0	" +
" 12	39.8	
" 13	39.6	
" 14	39.2	
" 15	39.2	
	39.0	

Preserved for immunity test with Nicolle's strain.

The four controls all had temperatures approaching or exceeding 40°C. or 104°F. on the 6th day, and between the 6th and 14th days two of them continued to run temperatures fluctuating between 40.3° and 40.8°C., or 104.5° and 105.5°F. The two others were used on the 10th day for transfers showing a fever of 40.6° and 40.8°C., respectively, when they were killed on the 5th day of continuous fever.

It is plain to be seen that the controls all passed through a typical typhus fever of the Nicolle type, whereas none of the flea animals developed a typhus reaction. It is obvious, therefore, that the animals which had been inoculated with a Mexican virus that had passed through fleas were immune to a subsequent inoculation with the Nicolle strain.

Transmission Experiments.—

These experiments were greatly hampered by an epizootic among our rats. In one rat a slightly positive Weil-Felix reaction was observed 15 days after infected fleas had started to feed on it. Unfortunately, the rat was found dead the next day, and the inoculation of its brain into a guinea pig produced peritonitis in the latter. The inoculation of Rats 4 and 10, respectively, was not followed by any symptoms.

Histological Examination of Fleas Fed on Typhus-Infected Rats

The following fleas were examined.

3 Xenopsyllae.—33 days after fever had been noticed in the inoculated rats, 29 days, respectively, after replacing the infected with a normal rat.

1 Xenopsylla.—13 days after the febrile period had started in the rats, 9 days, respectively, after the fleas had changed to a normal rat.

3 Leptopsyllae.—16 days after being put on infected rats.

2 Ceratophyllus fasciatus.—12 days after feeding on infected rats.

1 Ctenocephalus felis.—10 days after feeding on infected rats.

The fleas were killed by applying a drop of chloroform to the place where they were observed, and then carefully removed by a pair of soft forceps. The legs were clipped off as closely as possible to the body, then the head was removed with a razor blade, and finally the two last abdominal segments were cut off. The fleas were then fixed in Regaud's fluid for 12 hours, kept for 6 hours in sterile water, and then passed through the alcohol series, employing not more than 2 hours for this last step. After the absolute alcohol, they were transferred to oil of cedar wood, where they were kept for at least 24 hours before being transferred to paraffin of a melting point not less than 58°C. Serial sections 4 microns in thickness were cut through the whole length of the fleas. The sections were stained by the method of Wolbach, Todd and Palfrey, changing the Giemsa solution once and staining in the incubator for 12 to 16 hours. Alkalinization of the water was omitted. Perfect series of sections were obtained from several fleas. After fixation in Regaud's fluid, the *Rickettsiae* take an intensive purplish red color with Giemsa solution, and very little care has to be taken in differentiating the sections in 96 per cent alcohol, as the *Rickettsiae* retain the stain even better than most of the cellular structures of the insect's body.

The picture observed in the fleas was alike in all the species examined. The cells of the stomach and the epithelial lining of the Malpighian tubules are the sites of multiplication of *Rickettsia prowazecki* in the fleas. (By "stomach" we understand that part of the gut which is situated between the proventriculus and a place just in

front of the union of the four Malpighian tubules with the intestine.)
In the epithelial lining of the stomach, *Rickettsiae* settle first in the
basal part, where the cells adhere to the membrana propia. From
there, they spread to the rest of the cells, filling them in dense masses.
Such heavily infected cells protrude into the lumen of the stomach as
large, distally clubbed structures. In the fleas killed 16 days after
feeding on infected rats, only a few cells could be found which were
entirely free from infection, most of them showing a massive invasion
by *Rickettsiae*. The cells of the small intestine remain *free* of *Rickett-
siae*, with the exception of those which surround the opening of the
Malpighian tubules into the intestine. In comparing sections of
heavily infected lice with our sections of fleas, one is at once struck by
the large numbers of free *Rickettsiae* in the lice's guts and the enor-
mous swelling of the stomach cells in these insects. These free organ-
isms originate from the greatly swollen and disintegrating epithelial
cells. In our fleas, no such discharge of *Rickettsiae* into the lumen of
the gut could be observed. Indeed in perfect sections, with no indi-
cation of damage to the cells done during the process of sectioning,
only few organisms were seen free in the lumen of the stomach. It
was astonishing, also, that in the hind gut, which receives the discharge
of a stomach in which practically every cell was found to be infected,
no *Rickettsiae*, or at the most a few only, could be found. Only in a
specimen of *Xenopsylla cheopis* sectioned more than a month after
feeding on infected rats were fairly numerous *Rickettsiae* seen in the
hind gut near the opening of the Malpighian tubules. In one flea a
few *Rickettsiae* were observed in the lumen of the proventriculus and
in the distal part of the esophagus. The scarcity of *Rickettsiae* in the
contents of the stomach of the fleas can only be explained by the pres-
ence of a peritrophic membrane. This membrane covers the cylindri-
cal epithelium of the stomach as a well developed hyalin structure.
It is the continuation of the chitinous membrane which lines the esoph-
agus and the proventriculus (it may well be called chitinogenous
membrane). This membrane reaches distally to a place immediately
proximal to the union of the Malpighian tubules with the gut. Proxi-
mally, it increases slightly but steadily in thickness up to the corner
where the proventriculus joins the stomach. Here the membrane
assumes a decidedly chitinous aspect. Even here the cells beneath

the membrane may be heavily infected with *Rickettsiae*. There cannot be any doubt that this membrane prevents the discharge of organisms from the heavily infected cells into the lumen of the stomach. No organisms could ever be observed within this structure, suggesting a passage from the cells directly into the cavity of the stomach. How, then, did *Rickettsiae* gain entrance to the body of these cells, which are covered by a membrane apparently impermeable for particulate matter? We have already mentioned that the first signs of multiplication of them is noticed in the basal part of the cylindrical cells of the stomach, and not in the surface, which is covered by the peritrophic membrane. The same behavior of *Rickettsiae* is observed also near the union of the stomach with the esophagus, where this membrane already looks like chitin. *Rickettsiae* could not have entered the cells by penetrating the membrane here. As mentioned above, the peritrophic membrane reaches through the whole stomach to a place immediately in front of the union of the Malpighian tubules with the small gut. At this point, the heaviest infection of the cells of these tubules is noticed with regularity. From here, the infection spreads proximally into the stomach, each cell apparently infecting the next by contact beneath the peritrophic membrane. Distally, from the union of the gut with the Malpighian tubules, the epithelial cells of the gut remain free from *Rickettsiae*. Not all of the Malpighian tubules were found to be infected in each flea. When they were infected, however, the number of *Rickettsiae* within their cells decreased progressively from the place of their union with the gut, where the cells are greatly swollen and crowded with *Rickettsiae* to the extent of nearly blocking the lumen of the tubules up to their ends, where isolated groups only of organisms were observed in the protoplasm of the Malpighian cells. It is evident, therefore, that the number of *Rickettsiae* eventually excreted by the fleas' feces must depend more on the number of infected Malpighian tubules and on the intensity of their infection than on the degree and number of infected stomach cells, because the Malpighian cells are not covered by a membrane. Salivary glands, salivary ducts and the reproductive organs remain free from *Rickettsiae*.

A comparison of the sections of fleas killed between 12 and 16 days after feeding on infected rats with sections of fleas killed more than a

month after first feeding on infectious blood gave results which may prove of significance. Whereas in the 12 and 16 day fleas the infection of the cells with *Rickettsiae* was without exception extensive and evenly distributed over the whole stomach, in the latter the most distal part only showed heavy infection, and from this point the number of *Rickettsia*-infected cells decreased progressively up to the entrance into the esophagus. In addition to this, we observed a change in the configuration of the mucosa of the stomachs of the infected fleas. In many places the cylindrical epithelial lining was found to be replaced by masses of more or less concentrically arranged cells. Within these masses, mitotic cell division was repeatedly observed, but no signs of infection with *Rickettsiae* were seen. Undoubtedly these cell masses represent centres of rapid cell regeneration of the mucosa which had been partly destroyed by the infection. When one compares sections of normal fleas with sections of infected fleas, especially when the infection has been one of long standing, a considerable difference in the number of stomach cells is at once apparent in the two sets of fleas. Whereas in the normal stomach the cylindrical cells are densely packed, one pressing closely to the other, in the infected fleas the cells are more isolated, so that many have room enough to spread out more or less flatly on the membrana propria. Because of this, the lumen of the stomach in infected fleas is considerably wider.

Examination of Normal Fleas.—

From each lot of fleas which was to be used for our experiments, a number were reserved for microscopical examination, in order that we might become acquainted with the normal microbic flora.

Rickettsia prowazeki is easily recognized by the experienced by its morphology, and—more reliably—by its peculiar affinity for Giemsa's stain and for Castaneda's stain. When stained with Giemsa solution, the organisms stain red with a faint hue of purple, and with Castaneda's method they take a clear blue coloration. Ordinary bacteria do not stain blue by Castaneda's method, and with Giemsa solution they stain from blue to dark purple. This holds true when Giemsa stain is used for smears as well as for sections, provided that fixation is practiced in Regaud's fluid.

No organisms that took the coloration peculiar to *Rickettsia prowazeki* were observed in any of the normal fleas. *Rickettsia*-like microbes were observed in several fleas, especially in *Ctenocephalus*, but they were decidedly larger than

prevents the discharge of any considerable amounts of *Rickettsiae* into the lumen, in consequence of which, the flea feces are likely to be far less infectious than those of the lice. Again, we may assume that isolated flea bites are much less irritating to most human beings than are those of body lice, and lead to far less scratching and consequent inoculation. Again, the body of most fleas is much more strongly chitinized than that of lice, and in consequence the danger of crushing a flea against the skin is much less than is the case with lice.

The likelihood of transmission of the virus from rat to man in endemic regions by fleas is further diminished by the disinclination of the rat fleas *Xenopsylla* and *Ceratophyllus* for the human host. Although flea-infested rats have been handled by us almost every day in the course of this work, only once has a rat flea been found on an investigator, and on this occasion he had opened a glass container within which a rat had died during the night. Since typhus—in contradistinction to plague—does not kill rats under ordinary conditions, there is little occasion for the fleas to leave the normal host.

The variety of flea which is significant in the transmission of the virus from rat to man probably depends upon climatic conditions. In tropical and subtropical regions, the *Xenopsylla* varieties are probably the important ones, while in more northerly climates the significant fleas are probably *Ceratophyllus fasciatus*. *Ctenocephalus canis* and *Ctenocephalus felis* are probably of little importance, owing to their scarcity in rats. *Leptopsylla musculi* does not appear to infest man at all. In view of the ease with which we have been able to infect with the typhus virus all varieties of fleas with which we have worked, it is more than likely that *Pulex irritans* is no exception.[3] It is obvious, however, that this variety of flea cannot be of epidemiological importance; otherwise, it would be hard to understand why secondary infections are so rarely noticed in well managed hospitals and private houses, which cannot even with the greatest care be kept entirely free of fleas. Although, therefore, we can no longer maintain the rule cited by Otto and Munter, "without lice, no typhus," it is nevertheless still true that without lice there is no epidemic typhus.

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[3] Since this paper was written *Pulex irritans* has actually been infected by allowing it to feed upon human typhus cases and upon infected guinea pigs.

SUMMARY AND CONCLUSIONS

The virus of Mexican typhus has been shown to multiply abundantly in the following species of fleas: *Xenopsylla cheopis, Ceratophyllus fasciatus, Leptopsylla musculi, Ctenocephalus canis, Ctenocephalus felis.* In all fleas, *Rickettsia prowazeki* was demonstrated within the epithelial cells of the stomach and within the cells of the Malpighian tubules. Whereas in infected lice enormous numbers of these organisms are discharged from the disintegrating cells into the intestinal content, only few *Rickettsiae* are found in the lumen of the fleas' intestines. They are held back by the peritrophic membrane, which covers the mucosa of the entire stomach. *Rickettsiae* seem to enter the lumen of the gut almost exclusively by the route of the Malpighian tubules. Observations were made which seem to indicate that the fleas recover from the infection and that they are able to regenerate the partly destroyed intestinal mucosa. An explanation is given for the relative harmlessness of fleas as vectors of typhus.

We are indebted to Professor Hans Zinsser, of the Harvard University Medical School for many valuable suggestions. The drawings were made by Miss Etta Piotti.

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EXPLANATION OF PLATE 19

The fleas were fixed in Regaud's fluid and stained with Giemsa's solution.

FIG. 1. Cross-section through posterior abdominal end of *Xenopsylla cheopis.* One Malpighian tubule is infected with *Rickettsia prowazeki.* ×80.

FIG. 2. Heavily infected Malpighian tubule of the same flea at high power magnification. ×900.

FIG. 3. Same flea. Heavily infected cells of the stomach. Note the absence of organisms from the peritrophic membrane. ×900.

Malpighian tubules

Oviduct

Small intestine

Ovaries

Salivary glands

1

2

Peritrophic membrane

3

(Mooser and Castaneda: Virus of Mexican typhus fever in fleas)
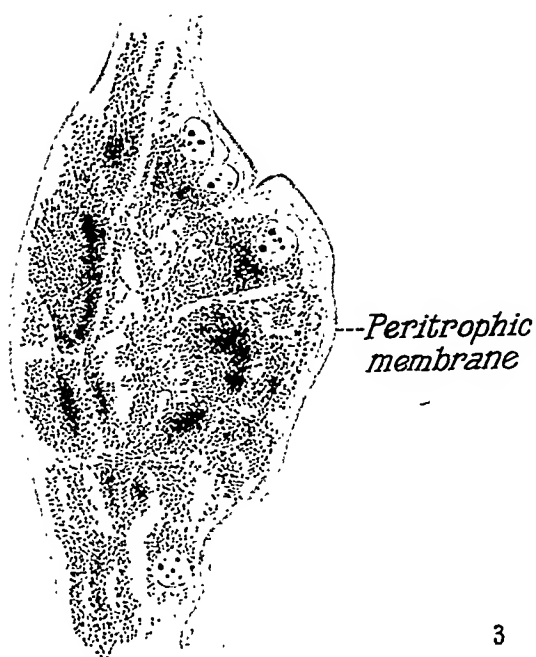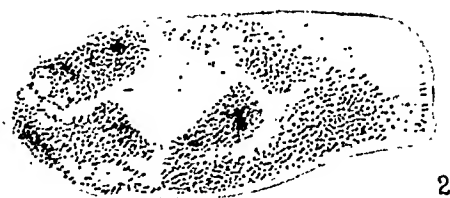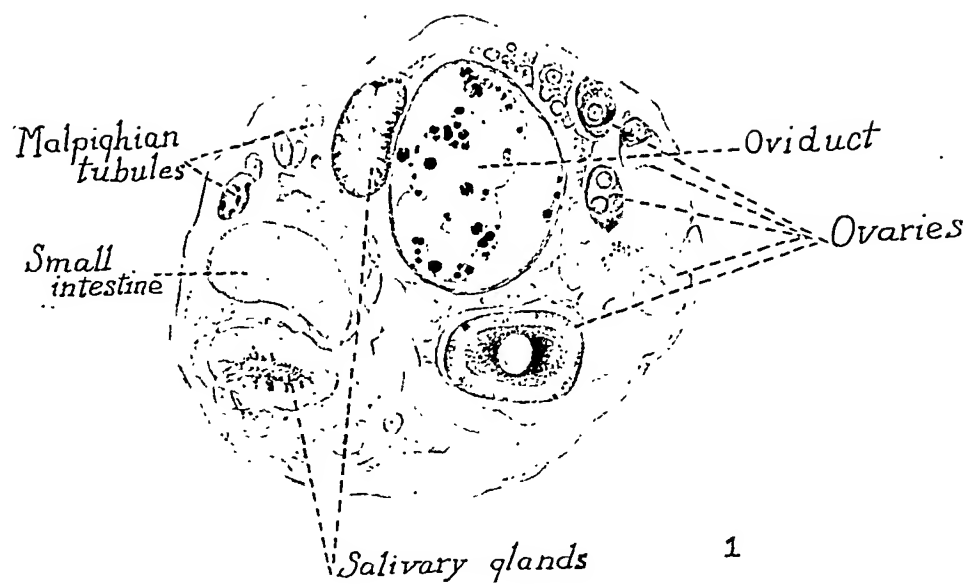
CARBOHYDRATES ADSORBED ON COLLOIDS AS ANTIGENS

By JOSÉ ZOZAYA, M.D.

(From the Mulford Biological Laboratories, Sharp and Dohme, Glenolden, Pennsylvania)

The phenomenon of adsorption has been used in biology for the purification of enzymes, toxins, etc., and offers an interesting and complicated mechanism, showing certain selectivity.

In the field of bacteriology and immunology many workers have used this phenomenon of adsorption in the study of diverse problems.

Nicolle (1) studying the agglutination reaction, grew different organisms (*B. proteus* and *B. typhosus*) in a filtrate of *B. coli*, and found that these organisms agglutinated with anticoli serum. He obtained similar results when talcum powder was used in the place of the organisms. These foreign particles, when placed in *B. coli* filtrate, were equally agglutinated by anticoli serum. He remarks on the constant presence of the agglutinable substance in the cultures of *B. coli*.

Arkwright (2) studying the agglutination of *B. typhosus* made use of *B. coli* and kieselguhr as indicators in showing the presence of agglutination reaction. When he added *B. typhosus* extract to *B. coli* emulsion, agglutination was observed. He says, "In this case the *B. typhosus* extract appeared to act as a 'protective colloid' and each particle (*B. coli* + *B. typhosus* extract) behaved as though it were composed of the extract. An adsorbed coating of each bacillus with *B. typhosus* colloid being assumed as probable."

Coulter (3) showed that red cells agglutinate at pH 4.75, but when sensitized with serum the agglutination point was shifted to that of globulin, pH 5.3. Northrop and De Kruif (4) found that a mixture of bacteria and egg albumin or bacteria and globulin behaved towards acid, like solutions of the respective proteins. The isoelectric point of the organism shifted to that of the added protein.

Loeb (5), working on cataphoresis with collodion particles showed the effect of electrolytes on them and concluded that they follow closely the same law that McTaggart (6) found for gas bubbles and water, giving the collodion particles a negative charge. Loeb (7) further demonstrated that the influence of different electrolytes was the same on gelatin-coated collodion particles, particles of casein and particles of boiled egg albumin. This fact showing that it is immaterial whether the protein is in the form of denatured egg albumin or a protein-like gelatin, furnishes proof that the solutions of genuine proteins are not diphasic

systems. Further experiments of Loeb (8) on the stability of suspensions of solid particles of proteins and the protective action of colloids showed that collodion particles, gelatin-coated, require the same salt concentration to precipitate them as did the "salting out" of gelatin from aqueous solutions.

Freund (9) studied physicochemical reactions of the agglutination of the tubercle bacillus. In this work he found that collodion particles are acid-fast when stained by the Ziehl-Neelsen method and that they remain acid-fast even if they are coated with protein.

Jones (10), studying the phenomenon of agglutination by precipitins, made use of the collodion particles and bacteria, sensitizing them and agglutinating them with the specific precipitin. He explained the results with heated (serum) antigens on the basis that coagulated proteins in suspension are covered with undenatured antigen and the addition of precipitin causes agglutination of the coagulated protein. He further noted that when particles were mixed with cow serum of crystallized egg albumin and then washed until free antigen no longer remained in the wash solution, they behaved like bacteria sensitized to cow serum and subsequently washed. In a later article (11) Jones showed that the amount of adsorption by the collodion particles, judged by agglutination, is not dependent on the concentration of the sensitized protein beyond a certain maximum, a significant observation, for Hitchcock (12) has shown that collodion membranes when in contact with solutions of crystallized egg albumin and gelatin adsorb the protein on their surfaces. The concentration of the protein, being in relation to the amount adsorbed, is expressed fairly well by the equation proposed by Langmuir (13) for the adsorption of gases by a plain surface. Jones also showed that particles exposed to a number of antigenic substances, crystallized egg albumin, rabbit serum, chicken serum, horse serum and cow serum, in succession, are equally agglutinated by all of the appropriate antisera.

Bedson (14) working with herpes virus, demonstrated that collodion particles adsorbed herpes virus or herpes antibodies. There is a definitely increased avidity for the virus in those particles which have been sensitized with herpes antiserum. The observation will be discussed further on.

Mudd, Lucké, McCutcheon and Strumia (15) have shown that collodion particles treated with precipitogen are phagocytized in a manner that is essentially identical with the phagocytosis of bacteria treated with bacteriotropins. They also found that particles so treated showed characteristic properties of protein, such as cohesiveness, wetting properties and were isoelectric at pH 5.5 to 5.8 and hence believe that precipitation, agglutination, the surface changes and increased phagocytosis are all consequences of a specific chemical combination with, and deposit on the surface of the antigen of antibody protein.

Rhoads (16) has adsorbed the virus of poliomyelitis on aluminum hydroxide particles and while he was unable to induce the disease by injection of the material, produced active immunity in *Macacus rhesus*. Carbohydrates as antigens have been studied, more especially since the isolation of the complex bacterial polysac-

charides. Wells (17) comments on carbohydrates as antigens, stating that there is no apparent theoretical reason why complex carbohydrates should not exhibit antigenic functions, as they may exist in the colloidal state. Nishimura (18) reports the result of his experiments on the production of specific antibodies, judged by the complement-fixation test with inulin, soluble starch and various dextrins. He concludes that in the production of immune bodies by these three kinds of polysaccharides, protein might play the part of the vehicle as all of them have a small percentage of N, probably in the form of protein. He noted that dextrin produced antibodies more readily when mixed with pig serum.

The use of bacterial polysaccharides for antibody formation has been attempted by many investigators. In the few successful cases the carbohydrate has usually been of doubtful purity. Thus the "residue-antigens" of Zinsser and Parker (19) were impure in some respects, Ross (20) found that rats fed Pneumococcus Type I polysaccharide in doses of 0.5 mg., were protected when tested with virulent pneumococci. Schiemann and Caspar (21) found mice to be more resistant when injected previously with a pneumococci specific substance free of protein, while in rabbits they were unable to induce agglutinins, precipitins or protective antibodies. They account for this failure by the probable insufficient dosage. Francis and Tillett (22) have found that patients injected intradermally with different polysaccharides from the pneumococci develop circulating antibodies these polysaccharides. They suggest that the development of these antibodies heterologous types of pneumococcus was associated with the previous intradermal injection of the type-specific polysaccharides. They believe it possible that in the process of recovery from infection, a highly reactive state exists in the human organism which responds to stimuli otherwise ineffective. Before one can accept this result, further work must be done.

Our work consists in the study of the antigenic potentialities of various polysaccharides and dextran adsorbed on colloids, especially on collodian particles. While the bacterial polysaccharides have not been free of nitrogen in any case, the significance of its presence in the preparation used for antibody production has always been controlled by injection into animals of the unadsorbed polysaccharide in dilutions of 1/10,000. The dextran was free of nitrogen and ash.

Method

Preparation of the Colloid Carrier.—Merck's C.P. collodion was used. This was precipitated in distilled water, washed several times and then dried between filter papers. The dry collodion was dissolved in warm acetone C.P. The acetone solution was placed in a jar and agitated fairly rapidly by an electric stirring machine. Water was slowly added to the collodion until a white heavy suspension of collo-

dion particles was noticed. The material was further diluted with distilled water until the larger clumps separated out. The acetone was removed by vacuum distillation at 45°C.

The large particles were removed by rapid centrifugation for 1 minute. The rest of the particles were obtained by further centrifugation for 10 minutes. There remained in the liquid a great many particles which were not brought down by centrifugation. These were obtained by adding to the liquid NaCl crystals, and the precipitated particles were centrifuged. All of the centrifuged particles were washed with physiological salt solution at least three times. The washed particles were kept in the ice box until used.

The casein used was prepared from skimmed milk by the method of Van Slyke and Baker. This was suspended in salt solution and used for adsorption in the same way as the collodion particles.

When organisms were used for adsorption they were washed in salt solution and the centrifuged material then used. We always used living organisms. The adsorption on them was carried out by the same method used with other particles.

Adsorption.—Solutions of the polysaccharide in 0.5 to 1 per cent concentration were used. To the centrifuged collodion particles 1 cc. of the polysaccharide solution was added and carefully mixed with a glass rod so as to avoid the formation of large clumps. The container was then placed on the ice for 10 minutes or at other times overnight (we observed no difference). Physiological salt solution was added and the particles washed four times. This is unnecessary when using non-antigenic substances, but was always done. After centrifuging, a small amount of salt solution was added very carefully and the collodion particles made into a homogeneous suspension. This suspension was standardized with a silica turbidity comparator to equal a suspension of *E. typhi* of 5,000,000,000 per cc. A few drops of chloroform were added to the so prepared antigen as preservative. When serum was adsorbed to the particles the same procedure was followed, the serum being diluted 1–10 and mixed with the collodion particles; after refrigeration, it was washed four times, and then the polysaccharide was adsorbed. In case of several polysaccharides, each was adsorbed separately to eliminate the error of dilution which would be present if they were mixed.

Immunization of Animals.—The rabbits were injected intravenously with 0.5, 1.0 and 1.0 cc. on consecutive days, followed by a 5 day rest. A second series of increasing doses were now given, and again a 5 day rest. Three injection series were completed before a trial bleeding. Horses were immunized by giving one injection a week. The doses varied between 25 and 250 cc.

Bacterial Polysaccharides.—The polysaccharides were prepared by various methods, some of which have been described in other publications. It is not necessary to give the chemical assay of each carbohydrate since adequate controls were made showing that the plain polysaccharide was not by itself antigenic. The ash content has no importance and fairly impure preparations may be used, as the adsorption technique in itself serves as a purifier of the substance.

In this group of experiments we have also used bacterial bodies as adsorbents. The results of the experiments will be given under the head of the polysaccharide adsorbed on them. The polysaccharides most used were those with which we have had the most experience, both in preparation and immunological reaction, namely those of certain Gram-positive bacilli, *B. anthracis* and *B. proteus;* the Gram-negative bacillus, *Bacterium morgani*; the Gram-positive cocci, the pneumococci and *Streptococcus viridans* (Bargen), and finally the Gram-negative coccus, the meningococcus.

Making of Tests. (a) *Polysaccharide Precipitin.*—The amount of the serum dilution used was usually 0.2 cc. and an equal amount of the polysaccharide solution. Mixed and incubated in the water bath at 37°C. The time of incubation was usually 2 to 4 hours. The test was then read and the tubes were placed in the ice box overnight when a second reading was made. In certain cases the tubes were centrifuged for a few minutes before the final reading was established.

The amount of precipitate and its quality (flocculent or disc formation) were estimated and called 4, 3, 2 or 1 according to the amount of precipitate in proportion to the dilution; 0 indicating no precipitate.

(b) *Horse Precipitin Tests.*—Equal amounts of test serum and antigen (horse or bovine serum diluted 1 to 10) were placed in contact in micro test-tubes and the amount of precipitate formed at the union of the two layers was read after standing 1 hour at room temperature as indicated by $+++$ (heavy and settling), $++$ (marked ring), $+$ (distinct ring), $\pm$ (faint), 0 (negative).

(c) *Agglutinations.*—Equal amounts of the serum dilution and antigen (2,000 million per cc. suspension of killed organisms) were mixed and incubated at 37°C. or 56°C. for various lengths of time from 2 to 18 hours, according to the type of organism used. The readings were made and tabulated as follows: 4 (complete agglutination), 3 (marked), 2 (less marked but definite), 1 (slight) and 0 (negative).

We shall first report the results with collodion particles as the colloid utilized in immunization; then we shall give the results using casein, then alumina and finally bacteria adsorbed with different polysaccharides. All the tests were *in vitro* tests, except the antipneumococcic in which mice were used.

Results of Immunization with Collodion Particles as the Colloid

Anthrax.—The sera of rabbits immunized with collodion particles and other combinations were studied for agglutinins, anti-horse precipitins and precipitins for the specific carbohydrate. The results are shown in Table I. The variations in the different groups immunized with collodion particles adsorbed with polysaccharide and other combinations do not show any marked difference from those in the

group (Rabbits 10, 11 and 12) immunized with the washed precipitate of antianthrax globulin + polysaccharide. Serum was used with the idea that it might increase the amount of polysaccharide adsorbed. The results with Rabbits 13 and 14 are given for the purpose of demonstrating the low antigenicity of the killed anthrax antigen. Rabbits 15 and 16 are only two from a group of ten, none of which showed any antibody response after prolonged treatment with the pure poly-

TABLE I

Agglutination, Horse Precipitin and Polysaccharide Precipitin Tests with the Sera of Rabbits Immunized with Different Combinations of Collodion Particles Adsorbed with Anthrax Polysaccharide

(Tests against same antigen, after three series of three injections each)

Immunized with...		Collodion particles + anthrax SSS			Collodion particles + anthrax globulin + anthrax SSS			Collodion particles + normal horse serum + anthrax SSS			Anthrax globulin + anthrax SSS precipitate			Anthrax organisms		Anthrax SSS	
Rabbit No........		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Aggluti- nation	1/4	4*	4	2	4	4	4	4	4	4	4	4	4	2	2	0	0
	1/8	4	3	1	4	4	4	4	4	2	4	4	4	1	2	0	0
	1/16	4	2	0	4	4	4	2	3	1	4	2	4	0	1	0	0
	1/32	4	0	0	2	4	2	1	2	0	1	0	3	0	0	0	0
	1/64	2	0	0	0	2	1+	0	0	0	0	0	1	0	0	0	0
Horse precipitin		0**	0	0	+	+	+	±	+	++	++	+++	+++	0	0	0	0
Bovine precipitin		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Anthrax SSS 1:1,000		1†	2	1	1	1	1	2	1	1	3	2	2	0	0	0	0

* Read after 15 hours at 37°C. 4 = complete; 3 = marked; 2 = less marked; 1 = slight; 0 = negative.

** Read after 1 hour at room temperature. +++ = heavy precipitate with settling; ++ = marked; + = distinct; ± = faint; 0 = negative.

† Polysaccharide precipitin test read after 4 hours.

saccharide. The horse precipitin test is of interest when we notice that only Rabbits 1, 2 and 3 of the first series were negative. Rabbits 13, 14, 15 and 16 were also negative. The polysaccharide precipitin test was weak, (1/4) but definite. Each rabbit had received approximately 0.03 mg. of the polysaccharide adsorbed on collodion particles. The results of the polysaccharide precipitin test are found at the bottom of Table I.

For comparison with the results obtained in rabbits, a horse (No. 1) was immunized with the same antigens using increasing doses, from 25 to 250 cc. Another horse (No. 2) was used as control, receiving equal amounts of a solution of polysaccharide 1/10,000, without collodion particles.

Keeping in mind that anthrax is a disease of horses and that any horse may have had the disease naturally or been artificially immunized, we tried the agglutination test before starting the immunization and found that No. 1 had none, while Horse 2 yielded a 3 agglutination in a 1/10 dilution. The result of the agglutination and polysaccharide precipitin test with the sera of these horses is shown in Table II.

TABLE II

Agglutination and Polysaccharide Precipitin Test with Serum from Horses Immunized with Collodion Particles Adsorbed with Anthrax Polysaccharide

Final dilution									
Test	Agglutination					SSS 1–1,000			
	1/10	1/20	1/40	1/80	1/160	1/2	1/4	1/8	1/16
Horse 1	4	4	3	2	0	3	3	2	1
Horse 2	3	2	0	0	0	0	0	0	0
Negative control	3	1	0	0	0	0	0	0	0

Read after 4 hours at 37°C.

At this time, we may mention the enormous difficulty encountered in finding a suitable antigen for the anthrax agglutination test. The majority of antigens give positive reactions with normal sera, and tests with freshly prepared antigens that have not been extensively controlled are unreliable.

To establish further the presence of antibodies in Horse 1 and the absence of them in Horse 2, we concentrated the serum with ammonium sulfate (usual method for concentration of antibacterial serum). The results of these agglutination and polysaccharide precipitin tests are shown in Table III.

In the agglutination test we have a very high titer for Horse 1, while in Horse 2, the titer is low. The agglutination titer noted in the latter horse, before we started treatment, was raised by concentration. The polysaccharide precipitin test was difficult to read on account of

the viscosity of the globulin (concentrated twenty-five times) and centrifugation was necessary before a clear reading was obtained.

TABLE III

Agglutination and Polysaccharide Precipitin Tests with the Serum Globulin Concentrated Twenty-Five Times of Horses 1 and 2 Which Had Been Immunized with Collodion Particles Adsorbed with Anthrax Polysaccharide

Test.............	Agglutination test									Polysaccharide precipitin test (1/10,000)							
Final dilution....	1/40	1/80	1/160	1/320	1/640	1/1,280	1/2,500	1/5,000	1/10,000	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
No. 1.............	4	4	4	4	4	4	4	4	2	4	4	4	4	4	3	2	0
No. 2.............	3	2	0	0	0	0	0	0	0	3	1	0	0	0	0	0	0
Control..........	2	1	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0

Read after 4 hours at 37°C.

TABLE IV

Agglutination (Type I), Horse Precipitin and Polysaccharide Precipitin Tests with the Serum of Rabbits Immunized with Meningococcus Polysaccharide Adsorbed on Collodion Particles

Immunized with.....	Collodion particles + meningococcus SSS		Collodion particles + antimeningococcus globulin + meningococcus SSS		Collodion particles + normal horse serum + meningococcus SSS			Meningococcus Type I organisms		Meningococcus SSS	
Rabbit No..........	1	2	3	4	5	6	7	8	9	10	11
Agglutina- 1/8	2*	0	3	2	0	3	1	2	0	0	0
tion 1/16	3	2	4	3	2	4	2	4	2	0	0
1/32	4	4	4	4	2	4	0	4	4	0	0
1/64	4	4	4	4	3	3	0	4	4	0	0
1/128	4	4	3	2	2	2	0	4	4	0	0
Horse precipitin	0**	0	++	+	+++	+	++	0	0	0	0
Bovine precipitin	0	0	0	0	0	0	0	0	0	0	0
Meningococcus SSS 1:1,000	0†	0	0	0	0	0	0	0	0	0	0

   * Read after 15 hours at 56°C.   ** Read after 1 hour at room temperature.
   † Read after 4 hours at 37°C.

Considering the concentration and the probable non-specific precipitation, a titer of 1/4 is very low and probably is non-specific when

one considers that the control globulin also gives a reading at this dilution.

We believe that these tests show conclusively, the non-antigenicity of our anthrax polysaccharide alone.

Meningococcus.—The polysaccharide used was a mixture of a polysaccharide prepared from a broad strain of meningococcus (Types I and III), and a small porportion of Types II and IV polysaccharides. At this time we believed all of the types to be immunologically similar. Table IV shows the results of the agglutination test with Type I meningococcus antigen. Table V gives the results of agglutination tests with three of the classical strains. A definitely higher aggluti-

TABLE V

Meningococcus Agglutination Test with the Sera of Rabbits Immunized with Collodion Particles Adsorbed with Meningococcus Polysaccharide

Immunized with.........	Collodion particles + meningococcus poly-saccharides						Controls					
Rabbit No.....	1			2			Positive serum			Negative serum		
Antigen.........	Type I	Type II	Type III	Type I	Type II	Type III	Type I	Type II	Type III	Type I	Type II	Type III
1/24	4	3	4	4	3	4	4	4	4	0	0	0
1/50	3	2	4	3	2	4	4	4	4	0	0	0
1/100	3	0	4	2	0	2	4	4	4	0	0	0
1/200	0	0	3	0	0	0	4	4	4	0	0	0

Read after 5 hours at 56°C.

nation titer against Types I and III is shown. At the moment we will merely present the results, which show the possibility of group specificity of the type polysaccharide when used as antigens. The polysaccharide precipitins appeared later, after another series of injections.

Horses were placed under treatment and the results of the tests will be given under the title of multiple antigens.

Bacterium morgani.—The results of the tests on immunized rabbits shown in Table VI are self-explanatory. The agglutinins are very high for this organism. We have no explanation for the negative

horse precipitin test except that the serum may not have been adsorbed on the collodion particle in Rabbits 3, 4, 5 and 6. Again we noted the negative polysaccharide test. On further immunization we obtained a strong reaction. Noticing the agglutinins increase rapidly we tested their specificity by absorbing with the homologous organisms; and in Table VII the results are shown. The absorption was complete after two trials, showing the specificity of the anticarbohydrate antibodies. This experiment was tried with the other antisera and

TABLE VI

Agglutination, Horse Precipitin and Polysaccharide Precipitin Tests on Serum of Rabbits Immunized with Morgani Polysaccharide Adsorbed to Collodion Particles

Immunized with......		Collodion particles + morgani SSS		Collodion particles + anti-morgani serum + morgani SSS		Collodion particles + normal horse serum + morgani SSS		S. morgani organisms		Morgani SSS	
Rabbit No............		1	2	3	4	5	6	7	8	9	10
Agglutina- tion	1/16	4*	2	4	4	4	4	4	4	0	0
	1/32	4	1	4	4	4	4	4	4	0	0
	1/64	4	0	4	4	4	4	4	4	0	0
	1/128	2	0	4	4	3	4	4	4	0	0
	1/500	0	0	4	4	0	4	4	4	0	0
	1/1,000	0	0	4	0	0	0	4	4	0	0
Horse precipitin Morgani SSS 1:1,000		0**	0	0	0	0	0	0	0	0	0
		0†	0	0	0	0	0	0	0	0	0

* Read after 2 hours at 56°C. ** Read after 1 hour at room temperature.
† Read after 4 hours at 37°C.

the results were similar. We give our results of the test with *Bacterium morgani* on account of the very high agglutination titer.

B. proteus.—The immunization of animals with this polysaccharide has been the least satisfactory in the production of agglutinins, when collodion particles were used as the colloid carrier. The results are shown in Table VIII. The negative horse precipitin test in Rabbits 3 and 4 is due to the use of rabbit antiserum. In the other cases in which we used specific antisera, horse serum was employed. The polysaccharide precipitin test was definite in these series of animals.

Streptococcus viridans (Bargen).—Table IX shows the results of

agglutination, polysaccharide precipitin and horse precipitin tests. It is of interest to note the variability in the response of animals to the treatment. We account for the higher antibody content in

TABLE VII

Absorption Tests with Bact. morgani Antigen in Sera Immunized with Bact. morgani Polysaccharide Adsorbed on Collodion Particles and Other Combinations

Immunized with.........	Collodion particles + *morgani* SSS						Collodion particles + anti-morgani serum + *morgani* SSS						Collodion particles + normal horse serum + *morgani* SSS					
Rabbit No.....	7			8			9			10			11			12		
Serum.......	Orig.	1st abs.	2nd abs.	Orig.	1st abs.	2nd abs.	Orig.	1st abs.	2nd abs.	Orig.	1st abs.	2nd abs.	Orig.	1st abs.	2nd abs.	Orig.	1st abs.	2nd abs.
1/4	4	2	0	4	0	—	4	1	0	4	2	0	4	4	0	4	4	0
1/8	4	1	0	4	0	—	4	0	0	4	1	0	4	2	0	4	3	0
1/16	4	0	0	4	0	—	4	0	0	4	1	0	4	1	0	4	0	0
1/32	4	0	0	3	0	—	4	0	0	4	0	0	4	0	0	4	0	0
1/64	4	0	0	2	0	—	4	0	0	4	0	0	4	0	0	4	0	0
1/128	2	0	0	1	0	—	4	0	0	4	0	0	4	0	0	4	0	0
1/256	2	0	0	0	0	—	2	0	0	2	0	0	3	0	0	4	0	0

Read after 2 hours at 56°C.

TABLE VIII

Agglutination, Horse Precipitin and Polysaccharide Precipitin Tests with Serum of Rabbits Immunized with Proteus Polysaccharide Adsorbed on Collodion Particles

Immunized with......		Collodion particles + *proteus* SSS		Collodion particles + anti-proteus serum + *proteus* SSS		Collodion particles + normal horse serum + *proteus* SSS		*Proteus* organisms		*Proteus* SSS	
Rabbit No...........		1	2	3	4	5	6	7	8	9	10
Agglutination	1/4	4*	4	4	4	3	3	4	4	0	0
	1/8	3	3	2	3	2	2	4	4	0	0
	1/16	2	0	1	0	1	0	4	4	0	0
	1/32	0	0	0	0	0	0	4	4	0	0
Horse precipitin		0**	0	0	0	++	+	0	0	0	0
Bovine precipitin		0	0	0	0	0	0	0	0	0	0
Proteus SSS 1:1,000		2†	3	2	3	2	1	4	4	0	0

* Read after 4 hours at 56°C. ** Read after 1 hour at room temperature.
† Read after 2 hours at 37°C. and overnight in ice box.

Rabbits 3, 4 and 5 on the basis that more polysaccharide had been adsorbed, for the amount of globulin adsorbed cannot in itself have been responsible. The protein alone does not account for the increased reaction, as Rabbits 7, 8 and 9 received collodion particles adsorbed with normal horse serum first.

TABLE IX

Agglutination, Horse Precipitin and Polysaccharide Precipitin Tests with the Serum of Rabbits Immunized with Strep. viridans (Bargen) Polysaccharide Adsorbed on Collodion Particles

Immunized with......		Collodion particles + Bargen SSS		Collodion particles + Bargen globulin + Bargen SSS			Collodion particles + normal horse serum + Bargen SSS			Bargen organism		Bargen SSS	
Rabbit No............		1	2	3	4	5	6	7	8	9	10	11	12
Agglutina- tion	1/4	2*	4	4	4	4	3	2	0	4	4	0	0
	1/8	1	3	4	4	4	2	0	0	4	4	0	0
	1/16	0	1	4	4	3	1	0	0	4	4	0	0
	1/32	0	1	4	4	2	0	0	0	4	4	0	0
	1/64	0	0	4	2	0	0	0	0	4	4	0	0
Horse precipitin		0**	0	+++	++	+++	++	++	+++	0	0	0	0
Bovine precipitin		0	0	0	0	0	0	0	0	0	0	0	0
Bargen SSS 1:1,000		1†	3	2	3	3	2	1	0	4	4	0	0

* Read after 2 hours at 56°C.   ** Read after 1 hour at room temperature.
† Read after 2 hours at 37°C.

TABLE X

Agglutination Test with Serum of Horse Immunized with Collodion Particles Adsorbed with Streptococcus (Bargen) Polysaccharide

Serum dilution.................................	1/2	1/4	1/8	1/16	1/32	1/64
Horse 50..	4	4	4	2	1	0
Normal horse serum...........................	0	0	0	0	0	0

Read after 2 hours at 56°C.

In view of these responses, we immunized a horse with collodion particles and Bargen polysaccharide. This animal received 855 cc. of suspension, equivalent in polysaccharide to about 1.9 mg. Tables X and XI show the agglutination and polysaccharide precipitin tests with the serum of this horse.

B. dysenteriae (Shiga and Hiss).—The polysaccharides from these two organisms were selected, first, because we had done considerable work with the Shiga variety, and second to determine whether the specific antibody found in antidysentery (Shiga) serum is an anti-carbohydrate antibody, or an antitoxin. These rabbits received 17 cc. of antigen which is equivalent to about 0.05 mg. of its respective polysaccharides.

TABLE XI

Polysaccharide Precipitin Test with the Serum of a Horse Immunized with Collodion Particles Adsorbed with Streptococcus (Bargen) Polysaccharide

Serum dilution	1/2	1/4	1/8	1/16	1/32	1/64
Horse 50	4	4	4	1	0	0
Normal horse serum	0	0	0	0	0	0

Read after 2 hours at 37°C. and overnight in ice box.

TABLE XII

Agglutination Tests with Serum of Rabbits Immunized with Collodion Particles Adsorbed with B. dysenteriae (Shiga) and B. dysenteriae (Hiss) Polysaccharides

Organism	B. dysenteriae (Hiss)						B. dysenteriae (Shiga)					
Serum dilution	1/50	1/100	1/200	1/400	1/800	1/1,600	1/2	1/4	1/8	1/16	1/32	1/64
(Shiga) No. 68	0	0	0	0	0	0	4	4	4	3	2	1
(Hiss) No. 70	4	4	4	4	4	3	0	0	0	0	0	0
Normal rabbit serum	0	0	0	0	0	0	0	0	0	0	0	0

Read after 2 hours in water bath at 56°C.

Table XII shows the agglutination test of the two test rabbits. It is of interest to note the specificity of each serum. In the preparation of agglutinating antiserum we always get cross-reactions with Shiga and Hiss types. We cannot account for the difference in titer of the two types of sera, except by supposing that one (Hiss) might be more easily adsorbed. Tests in mice are under way to see if the toxin prepared from the organisms is neutralized by this antiserum.

Pneumococci.—Consistent failure has been the result in our hands of immunization experiments with these polysaccharides. This in-

cludes Type I which seems to contain normally about 5 per cent nitrogen, though this is not in the form of protein.

We have carried out immunization experiments in horses and rabbits with the object of producing immune serum, and have used mice for experiments on active immunity. Our first attempt was to immunize mice with Pneumococcus Types I, II and III polysaccharides adsorbed on collodion particles. A control series was injected with

TABLE XIII

Virulence Test on Mice Injected with 0.5 Cc. Intravenously and Intraperitoneally of a Suspension of Collodion Particles Adsorbed with Pneumococcus Polysaccharide Types I, II and III Separately

Type of organism...	Pneumococcus Type I		Pneumococcus Type II		Pneumococcus Type III	
Route of injection....	Intravenous	Intraperitoneal	Intravenous	Intraperitoneal	Intravenous	Intraperitoneal
cc.						
10⁻²	18 18	— —	— —	— —	— —	— —
10⁻³	18 S	18 24	18 24	18 18	— —	— —
10⁻⁴	S S	24 S	18 18	18 72	18 18	24 48
10⁻⁵	S S	24 S	24 36	24 S	24 36	S S
10⁻⁶	S S	S S	S S	S S	24 72	S S
10⁻⁷	24 48	24 96	24 36	72 S	24	48
Control......	Virulence	Polysaccharide	Virulence	Polysaccharide	Virulence	Polysaccharide
cc.						
10⁻⁹	24 24	72 —	24 36	S —	36 S	48 48
10⁻⁸	22 22	36 36	24 24	36 —	36 36	24 48
10⁻⁷	18 36	36 —	24 36	24 —	24 36	48 —

S = survival; numerals indicate the number of hours elapsing before death.
— = not done.

polysaccharide alone. One injection was given to two series of mice, in one intravenously and in the other intraperitoneally. 7 days later these mice were tested for immunity against various amounts of live, virulent cultures of pneumococcus. The result of our first test is given in Table XIII. Encouraging results were observed, and further work is being done with the object of working with other colloids as well as of determining the optimum time factor and dosages.

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We then proceeded with the immunization of horses with collodion particles adsorbed with Types I, II and III polysaccharides respectively. (The Type III polysaccharide used was kindly supplied to us

TABLE XIV

Homologous Polysaccharide Precipitin Test with the Sera of Horses Immunized with Collodion Particles Adsorbed with Types I, II and III Pneumococcus Polysaccharides

Dilution of serum..	1/2	1/4	1/8	1/16	1/32
Type I Horse 49	3	2	0	0	0
Type II Horse 52	3	3	2	1	0
Type III Horse 87	3	3	2	2	0

Read after 3 hours at 37°C. and overnight in ice box.

TABLE XV

Protection Test in Mice with Serum of Horses Immunized with Collodion Particles Adsorbed with Pneumococcus Polysaccharides

Type..........	Pneumococcus I		Pneumococcus II		Pneumococcus III	
Horse No......	49		52		87	
cc.						
10^{-8}	S					
10^{-7}	S	S				
10^{-6}	72	S	S			
10^{-5}	48	S	S	S	36	36
10^{-4}	48	S	S	S	36	36
10^{-3}	48	72	S	S	24	36
			S	S	24	36
			24	24	24	36
						24
Virulence						
10^{-9}	48	S	24	S	96	S
10^{-8}	48	48	24	24	24	24
10^{-7}	24	24	24	24	24	24

S = survival. Numerals indicate the number of hours elapsing before death.

by Dr. M. Heidelberger.) Horse 49 was immunized against Type I, having received during 2 months 855 cc. of the antigen, approximately 2 mg. in terms of polysaccharide. Horse 52 was immunized against Type II, receiving during 4 weeks 425 cc. of antigen, or about 0.9

mg. in terms of polysaccharide. And finally, Horse 87, immunized against Type III, received in 4 weeks 425 cc. of antigen or 0.9 mg. of polysaccharide. 1 day after the last injection, the horses were trial bled, and agglutination, polysaccharide precipitin and protection tests were made. Tables XIV and XV show the results of these tests. We did not observe any agglutination with any of the types. At this time Type III antiserum does not show any protection although it clearly gives a precipitin test at a dilution of 1/16 of serum. The immunization of the horses is being continued.

The result obtained with Type III polysaccharide immunization is not surprising when one considers the difficulty experienced in pro-

TABLE XVI

Agglutination and Polysaccharide Precipitin Test with Sera of Rabbits Immunised with Collodion Particles Adsorbed with Anthrax, Meningococcus and Bargen Polysaccharides, at the Same Time

Antigen..........	Anthrax			Meningococcus Type I			*Strep. viridans* (Bargen)			
Rabbit No........	1*	2	3	1**	2	3	—*	1	2	3
1/20	4	4	4	3	4	4	1/2	4	2	4
1/40	3	1	2	1	2	4	1/4	2	0	3
1/80	1	0	0	0	0	4	1/8	1	0	3
1/160	0	0	0	0	0	2	1/16	0	0	1
SSS 1–1,000	0†	0	±	1	1	3	—	3	2	2

* Read after 2 hours at 56°C.   ** Read after 15 hours at 56°C.
† Read after 2 hours at 36°C. and overnight in ice box.

ducing protective serum even when the whole Type III pneumococcus organism is used for immunization. In the treatment of horses, more than 6 months is usually necessary before any definite protection is shown in their serum.

Multiple Polysaccharides Adsorbed on Collodion Particles

Having successfully used as antigens, collodion particles adsorbed with single polysaccharides, we now adsorbed different polysaccharides on the same particles. Table XVI shows the results of agglutination and polysaccharide precipitin tests on the sera of rabbits immunized with collodion particles adsorbed with three distinct polysaccharides.

The variations observed in the different rabbits are probably due to individual variation in the response of the animal to the antigen.

Table XVII shows the results of agglutination tests with serum from Horse 73 which received 460 cc. of collodion particles adsorbed with

TABLE XVII ·

Agglutination with Anthrax, Meningococcus Type I, Pneumococcus Type I and Streptococcus (Bargen) with Serum of Horse 73 Immunized with Collodion Particles Adsorbed with Polysaccharides of Same Organisms

Final dilution	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Anthrax	4*	4	4	4	3	1	0
Meningococcus Type I	4**	4	4	3	1	0	0
Pneumococcus Type I	0†	0	0	0	0	0	0
Streptococcus (Bargen)	4*	4	3	0	0	0	0

    * Read after 2 hours at 56°C.   ** Read after 15 hours at 56°C.
    † Read after 2 hours at 37°C.

TABLE XVIII

Protection Test in Mice with Serum of Horses Immunized with (72) Casein and (73) Collodion Particles Adsorbed with Anthrax, Meningococcus, Streptococcus (Bargen) and Pneumococcus Type I Polysaccharides

Organism	Type I pneumococcus			
Horse No.	72		73	
cc.				
10^{-8}	S	S	S	S
10^{-7}	S	S	S	40
10^{-6}	S	S	S	40
10^{-5}	S	S	40	40
10^{-4}	S	S	24	40
Virulence				
10^{-9}	72	S	—	—
10^{-8}	40	40	—	—
10^{-7}	24	40	—	—

    S = survival after 96 hours.   Numbers indicate hours elapsing before death.

anthrax, meningococcus, streptococcus (Bargen) and Pneumococcus Type I polysaccharides. The agglutination test for pneumococcus was negative, so mouse protection tests were made with virulent organisms, the result of the test being shown in Table XVIII.

mg. in terms of polysaccharide. And finally, Horse 87, immunized against Type III, received in 4 weeks 425 cc. of antigen or 0.9 mg. of polysaccharide. 1 day after the last injection, the horses were trial bled, and agglutination, polysaccharide precipitin and protection tests were made. Tables XIV and XV show the results of these tests. We did not observe any agglutination with any of the types. At this time Type III antiserum does not show any protection although it clearly gives a precipitin test at a dilution of 1/16 of serum. The immunization of the horses is being continued.

The result obtained with Type III polysaccharide immunization is not surprising when one considers the difficulty experienced in pro-

TABLE XVI

Agglutination and Polysaccharide Precipitin Test with Sera of Rabbits Immunised with Collodion Particles Adsorbed with Anthrax, Meningococcus and Bargen Polysaccharides, at the Same Time

Antigen..........	Anthrax			Meningococcus Type I			Strep. viridans (Bargen)			
Rabbit No........	1*	2	3	1**	2	3	—*	1	2	3
1/20	4	4	4	3	4	4	1/2	4	2	4
1/40	3	1	2	1	2	4	1/4	2	0	3
1/80	1	0	0	0	0	4	1/8	1	0	3
1/160	0	0	0	0	0	2	1/16	0	0	1
SSS 1–1,000	0†	0	±	1	1	3	—	3	2	2

* Read after 2 hours at 56°C. ** Read after 15 hours at 56°C.
† Read after 2 hours at 36°C. and overnight in ice box.

ducing protective serum even when the whole Type III pneumococcus organism is used for immunization. In the treatment of horses, more than 6 months is usually necessary before any definite protection is shown in their serum.

Multiple Polysaccharides Adsorbed on Collodion Particles

Having successfully used as antigens, collodion particles adsorbed with single polysaccharides, we now adsorbed different polysaccharides on the same particles. Table XVI shows the results of agglutination and polysaccharide precipitin tests on the sera of rabbits immunized with collodion particles adsorbed with three distinct polysaccharides.

JOSÉ ZOZAYA

The variations observed in the different rabbits are probably due to individual variation in the response of the animal to the antigen.

Table XVII shows the results of agglutination tests with serum from Horse 73 which received 460 cc. of collodion particles adsorbed with

TABLE XVII

Agglutination with Anthrax, Meningococcus Type I, Pneumococcus Type I and Streptococcus (Bargen) with Serum of Horse 73 Immunized with Collodion Particles Adsorbed with Polysaccharides of Same Organisms

	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Final dilution					3	1	0
	4*	4	4	4	1	0	0
Anthrax	4**	4	4	3	0	0	0
Meningococcus Type I	0†	0	0	0	0	0	0
Pneumococcus Type I	4*	4	3				
Streptococcus (Bargen)							

** Read after 15 hours at 56°C.

* Read after 2 hours at 56°C.
† Read after 2 hours at 37°C.

TABLE XVIII

Protection Test in Mice with Serum of Horses Immunized with (72) Casein and (73) Collodion Particles Adsorbed with Anthrax, Meningococcus, Streptococcus (Bargen) and Pneumococcus Type I Polysaccharides

	Type I pneumococcus			
Organism				73
Horse No	72		73	
cc.				S
			S	40
		S	S	40
10⁻⁸	S	S	S	40
10⁻⁷	S	S	40	40
10⁻⁶	S	S	24	
10⁻⁵	S	S		
10⁻⁴	S			
			—	—
Virulence		S	—	—
10⁻⁹	72	40	—	—
10⁻⁸	40	40		
10⁻⁷	24			

S = survival after 96 hours. Numbers indicate hours elapsing before death.

anthrax, meningococcus, streptococcus (Bargen) and Pneumococcus Type I polysaccharides. The agglutination test for pneumococcus was negative, so mouse protection tests were made with virulent organisms, the result of the test being shown in Table XVIII.

Results of Immunization with Aluminum Hydroxide as Colloid.—We adsorbed alumina with different polysaccharides, first one at a time, then two and three. The results of the multiple polysaccharide adsorption given in Table XIX suffice to show the success obtained.

TABLE XIX

Agglutination Test with Anthrax, Meningococcus Type I and Streptococcus (Bargen) of Serum of Rabbit 42, Immunized with Aluminum Hydroxide Adsorbed with Polysaccharides of the Same Organisms

Final dilution	1/4	1/8	1/16	1/32	1/64	1/128	SSS 1–1,000
Anthrax	4*	4	4	4	4	2	3
Meningococcus Type I	4**	4	3	1	0	0	2
Streptococcus (Bargen)	4*	2	1	0	0	0	2

* Read after 2 hours at 56°C.   ** Read after 15 hours at 56°C.

TABLE XX

Homologous Agglutination Test with Sera of Rabbits Immunized with Casein Adsorbed with Morgani, Bargen and Anthrax Polysaccharides, Separately

Antigen	Casein + *morgani* SSS			Casein + Bargen SSS			Casein + anthrax SSS		
Rabbit No	64	65	—	69	70	71	66	67	68
1/16	4	4	—	4	2	1	2	4	2
1/32	4	4	—	4	1	0	1	4	1
1/64	4	4	—	3	0	0	0	4	0
1/128	3	4	—	1	0	0	0	4	0
1/256	3	4	—	0	0	0	0	2	0
Milk agglutinins	+	+	—	+	+	+	+	+	+

Read after 2 hours at 56°C.

We have successfully immunized a horse with alumina adsorbed with meningococcus polysaccharide and another with alumina adsorbed with anthrax, streptococcus (Bargen), meningococcus and Pneumococcus Type I polysaccharide.

Results of Immunization with Casein as Colloid.—The use of the protein, casein, as the colloid carrier is of importance as showing that a protein carrier can effectively adsorb polysaccharides, the combina-

TABLE XXI

Agglutination with Sera of Rabbits Immunized with Casein Particles and Bacterium morgani, B. anthracis and Streptococcus (Bargen) Polysaccharides Together

Immunized with.......	Casein + *Bacterium morgani, B. anthrax* and Streptococcus (Bargen) SSS								
Rabbit No...	60			61			62		
Antigen......	*Morgani*	Bargen	Anthrax	*Morgani*	Bargen	Anthrax	*Morgani*	Bargen	Anthrax
1/4	4	0	4	4	2	4	4	3	4
1/8	4	0	4	4	1	4	4	2	4
1/16	3	0	3	4	0	2	4	1	4
1/32	2	0	2	4	0	1	4	0	2
1/64	1	0	1	4	0	0	4	0	1
1/128	0	0	0	4	0	0	4	0	0
Milk agglutination	4			4			4		

Positive and negative controls as in previous table.   Read after 2 hours at 56°C.

TABLE XXII

Agglutination and Polysaccharide Precipitin Tests with Anthrax, Meningococcus Types I, II, III, IV and V, Pneumococcus Type I and Streptococcus (Bargen) with the Serum of Horse 72, Immunized with Casein Adsorbed with Polysaccharide from the Same Organisms

Test	Agglutination						Polysaccharide precipitin test (1/10,000)		
Final dilution.........................	1/4	1/8	1/16	1/32	1/64	1/128	1/2	1/4	1/8
Anthrax.................................	4	4	4	4	2	1	4*	2	0
Pneumococcus Type I....................	0	0	0	0	0	0	3	2	1
Streptococcus (Bargen).................	4	4	2	1	0	0	4	1	0
Meningococcus Type I*..................	4	4	4	4	4	4	4	2	0
Meningococcus Type II..................	4	4	4	4	3	2	—	—	—
Meningococcus Type III.................	4	4	4	3	1	0	—	—	—
Meningococcus Type IV.................	4	3	1	0	0	0	—	—	—
Meningococcus Type V..................	4	4	4	1	0	0	—	—	—

* Precipitin tests read after 2 hours at 37°C. and overnight in the ice box.
** Tests for agglutination with meningococcus read after 15 hours at 56°C., all the others after 2 hours at 37°C.

tion being antigenic.   Avery and Goebel have made use of proteins, combining them chemically with polysaccharides, but we used casein

adsorbed with various polysaccharides.   Table XX shows the results of agglutination tests with sera of animals immunized with casein adsorbed with anthrax, *morgani* and Bargen polysaccharides respectively, and Table XXI shows the results of the agglutination test with the serum of animals immunized with casein adsorbed with the same three polysaccharides at the same time.

Horse 72 was immunized with casein adsorbed with meningococcus, Pneumococcus Type I, streptococcus (Bargen) and anthrax polysaccharides.   The results of the agglutination test with the serum of this

TABLE XXIII

Agglutination and Polysaccharide Precipitin Tests with Sera of Rabbits Immunized with Streptococcus (Bargen) Adsorbed with Different Polysaccharides

Immunized with..	Bargen organisms + *proteus* SSS				Bargen organisms + *morgani* SSS				Bargen organisms + anthrax SSS			
Rabbit No.......	78		79		80		81		82		83	
Antigen........ {	*Proteus*	*Bargen*	*Proteus*	*Bargen*	*Morgani*	*Bargen*	*Morgani*	*Bargen*	*Anthrax*	*Bargen*	*Anthrax*	*Bargen*
1/4	4	4	2	4	4	4	4	4	4	4	4	4
1/8	4	4	1	4	4	4	4	4	4	4	2	4
1/16	4	4	0	4	4	4	4	4	2	4	0	4
1/32	4	4	0	4	4	4	4	4	0	4	0	4
1/64	3	4	0	4	4	4	4	4	0	4	0	4
1/128	0	4	0	4	4	4	4	4	0	4	0	4
SSS 1–1,000	2	4	2	3	0	4	1	4	0	4	0	4

Read after 2 hours at 56°C.

horse is shown in Table XXII.   The pneumococcus did not show any agglutination.   The mouse protection test against virulent Type I pneumococcus is shown in Table XVIII.   The polyvalency against meningococcus is of interest.

Results of Immunization with Bacterial Cells as Colloids.—Before attempting immunization experiments with adsorbed polysaccharides on bacteria, we made a few preliminary experiments *in vitro*.   We selected *Streptococcus viridans* (Bargen) as the bacterium.   It was grown in Douglas media for 24 hours, centrifuged and washed once with saline.   To the centrifuged cells the polysaccharide was added and thoroughly mixed.   We allowed this to stand in the ice box for ½

hour, after which it was washed four times with saline. The last washing was free from detectable amounts of polysaccharide. These organisms were suspended in saline in a concentration of about 2,000 billion, and agglutination tests were made with serum homologous to the adsorbed polysaccharide with positive results. This no doubt was the phenomenon observed by Nicolle, Arkwright and Jones in their studies on agglutination. Having successfully repeated this with many different polysaccharides, we began the immunization of

TABLE XXIV

Agglutination and Polysaccharide Precipitin Tests with Sera of Rabbits Immunized with Bargen Organisms Adsorbed with Two Different Polysaccharides on the Same Cell

Immunized with..	Bargen organisms + anthrax and *proteus* SSS			Bargen organisms + *morgani* and anthrax SSS			Bargen organisms + Pneumococcus Type I and *morgani* SSS		
Rabbit No.......	77			75			84		
Antigen.........	Anthrax	*Proteus*	Bargen	Anthrax	*Morgani*	Bargen	Pneumococcus Type I	*Morgani*	Bargen
1/4	4	4	4	4	4	4	0	4	4
1/8	4	4	4	4	4	4	0	4	4
1/16	4	2	4	4	4	4	0	4	4
1/32	2	0	4	4	4	4	0	4	4
1/64	0	0	4	2	4	4	0	4	4
SSS 1–1,000	0	3	4	0	2	0	0	2	4

Read after 2 hours at 56°C.

animals. The results of our tests with different polysaccharides are shown in Table XXIII.

We then adsorbed more than one polysaccharide to the bacterial cell and immunized animals. The results of our tests are shown in Table XXIV. It is of interest to note that the specific antigen of the cell remained the most prominent of all both in agglutinins and precipitins. The streptococci adsorbed with Pneumococcus Type I polysaccharide agglutinated *in vitro*, but did not produce agglutinins against the pneumococci when used as antigen. Unfortunately this serum was mislaid and we were unable to make protection tests. Comparing these results with our previous experiments with pneumo-

conclusively that the precipitate formed by the immune serum and the dextran polysaccharide is specific. Methylated dextran as antigen does not give cross-reactions with this serum.

In a separate article we are reporting the relation of the active group or groups of dextran to some bacterial polysaccharides.

We attempted to immunize animals with levan, a polymerization product of an anhydro-fructo-furanose, produced synthetically from sucrose by *B. mesentericus* or its enzyme, but with negative results so far. Either the active group of levan is attached to the colloid or the antibodies produced were not to be detected by our methods of investigation.

DISCUSSION

Bacteria, being living organisms, have protoplasm, and this protoplasm always contains protein. It has been inferred that antigens must be of protein nature, and that the protein must have a certain complexity. The generalization now seems warranted that this need not be the case, but that some substance of a colloid nature is responsible for antigenicity (*vide* Willstätter's theory of enzyme action (23)). The colloid may be in certain cases a protein, but other forms of colloids which can adsorb the specific principle can suffice instead. The physical attachment makes the combination an antigen. In some cases a carbohydrate, in other cases a lipoid and in yet others a specific nitrogenous (protein) radical give to the antigens their specificity. In the case of pure proteins, we no doubt have a special radical in the whole molecule which is the active group and gives the antibody its specificity. Cross-reactions with antibodies may be explained by chemical similarity in the structure of the active group, an idea suggested by the work of Heidelberger and Avery (24) with the *B. friedlaenderi* and Pneumococcus Type II polysaccharide as well as by the work of Avery and Goebel (25) with gluco- and galacto-albumin and globulin, as also by the work of Obermeyer and Pick, (26) who studied the changes in specificity brought about by subjecting proteins to various chemical reactions, such as that caused by iodine, or nitric acid, the chemical changes thus induced giving rise to new antigens with altered serological properties.

We have shown in this work that dextran, a polysaccharide free of

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nitrogen, can become antigenic when adsorbed on collodion particles. The experiments with the antiserum thus obtained demonstrate that carbohydrate antibodies are specific. They can be of high titer, as judged by the precipitin test and by the agglutination test. From the theoretical side it is important to note our failure to obtain positive agglutination reactions with various dilutions of the polysaccharide and collodion particles adsorbed with immune serum. Our belief is that the adsorption of the immune serum to the particle comes about by means of the groups which have specific attraction to the polysaccharide, the hydrophilic groups being left free in this case. The polysaccharide, in solution with water, has the hydrophilic groups orientated towards the water phase. If this is so, one could not expect attraction towards the serum adsorbed on the particle. In the case of the particle adsorbed with the polysaccharide, one may suppose on the evidence of specific antigenicity that orientation occurs in such a way so as to leave the active groups free for a meeting with the specific group of the serum. The serum being attached to the water phase by the hydrophilic group leaves the specific group free to be attached to the adsorbed polysaccharide, causing agglutination.

In another communication, suggestive evidence is reported of the existence of various active groups in one bacterial polysaccharide.

Of theoretical interest are the calculations of the small amounts of polysaccharide needed to produce antibodies, especially in horses, in which 0.5 to 1.0 mg. was sufficient to give a fair titer to the serum. How much of the response in the animal is caused by the polysaccharide-colloid combination and how much is due to the exceedingly small amount of the polysaccharide alone, remains to be definitely determined. We are unable to tell the duration of the immunity produced by the polysaccharide or the degree of hyperimmunization which can be obtained. These points are being studied. There is no doubt that antibodies are detectable a short time after the injection and we have also observed the disappearance of the precipitin antibody on prolonged immunization. We have not been able to explain the latter phenomenon.

The extent of the practical application of our work, experience will decide. The advantages of an antiserum free from protein antibodies would seem plain. It is conceivable that one may prepare specific

bacterial polysaccharides and other antigenic haptenes for use in active immunization against infectious diseases in which the polysaccharides of the organisms play a part. The multiple nature of the haptogens in one particle has many attractive possibilities. The adsorption method may help the chemist to determine active groups in substances which are difficult to analyze by the methods now at hand.

SUMMARY

Evidence is here given that polysaccharides can be rendered antigenic by haptogenic adsorption upon a colloid carrier. The polysaccharides studied were those of B. anthracis, the meningococcus, Streptococcus viridans (Bargen), B. proteus, S. morgani, B. dysenteriae, both the Shiga and Hiss types, and the pneumococci. With the polysaccharide of Type III pneumococci, we have been unable in 6 weeks to produce any detectable protective antibodies, but we were able to produce anticarbohydrate antibodies. All the bacterial carbohydrates were non-antigenic alone when used in the doses indicated, though containing some nitrogen. Dextran, which was free from nitrogen was also rendered antigenic by the adsorption method.

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IMMUNOLOGICAL REACTIONS BETWEEN DEXTRAN POLYSACCHARIDE AND SOME BACTERIAL ANTISERA

By JOSÉ ZOZAYA, M.D.

(From the Mulford Biological Laboratories, Sharp and Dohme, Glenolden, Pennsylvania)

The name dextran was given by Scheibler (1) to the mucous fermentation products present in beet juices. He found that this substance on hydrolysis yielded considerable quantities of glucose and when oxidized with nitric acid gave oxalic acid. He was led to regard it as an anhydride of glucose.

Many other descriptions of this and similar substances are given in literature, but at present it is generally accepted that the production of the gum is due to the action of bacteria on rich sugar media. Depending on the kind of sugar and the type of organism used, different polysaccharides are obtained. In the case of gum-producing bacteria, large amounts of polysaccharides are produced because the nutrient medium used is rich in sugar. Boas (2) recommended a culture medium containing from 5 to 10 per cent of carbohydrate. According to the commonly accepted theory, it is assumed that most, if not all, carbohydrate-decomposing microorganisms possess synthesizing properties which, during the early stages of their development give rise under favorable conditions to the condensation of hexoses and other simple sugars to complex polysaccharides. In cases in which these synthetic polysaccharides readily imbibe water, a colloid, i.e., a mucus, is formed.

The most important of the bacteria capable of producing dextran from saccharose is *Leuconostoc mesenterioides*. The method of preparation, purification and structure of the dextran produced by this organism will be found described in forthcoming papers (3).

The material used in our experiments was kindly supplied by Prof. Hibbert and shown by analysis to be nitrogen- and ash-free.

An antidextran serum has been prepared by the author by adsorbing the dextran on collodion particles utilizing a method reported in a recent paper (4). The relation between the dextran polysaccharide

TABLE I

Precipitin Test with Dextran Polysaccharide and Various Antibacterial Sera

Serum	Dextran 1-5,000	Serum	Dextran 1-5,000	Serum	Dextran 1-5,000
S. paratyphi A	4*	Pneumococcus Type III	3	Strep. faecalis (1914)	1
S. suipestifer	4	Pneumococcus Type II	4	Enterococcus (1917)	4
Strep. haemolyticus	0	Pneumococcus Type I	4	Strep. ulcer. colitis type (1838)	0
S. morgani	4	Strep. ulcer. colitis type (1915)	0	Strep. ulcer. colitis type (1916)	4
E. typhi	4	B. dysenteriae (Shiga)	0	S. aertrycke	4
S. schottmuelleri	0	B. dysenteriae (Hiss)	4	B. proteus	0
Strep. haemolyticus (S.F.)	2−	B. anthracis	0	B. tuberculosis	0
Strep. haemolyticus (erysipelas)	0	Meningococcus polysaccharide	0	S. gallinarum	4
Strep. viridans	0	Anti-R I pneumococcus	4	Strep. ulcer. colitis (1918)	3
Strep. cardioarthritidis	0	Anti-R II pneumococcus	4	Strep. poliomyelitis	0

Read after 5 hours at 37°C.—overnight in ice box.

* 4 = definite large floccules settling in a disc; 3 = smaller floccules settling; 2 = definite fine precipitate; 1 = very slight fine precipitate; 0 = negative.

and various bacterial antisera has now been investigated and various interesting cross-reactions have been obtained while testing the specificity of this polysaccharide. The bacterial antisera utilized in the work were all prepared by injecting rabbits with whole formalinized culture of the organisms. The injections were given intravenously and treatment continued until tests on trial bleeding showed a definite, specific polysaccharide precipitate. In a few cases antisera produced in horses by a similar technique were employed.

TABLE II

Titration with Dextran Polysaccharide against Several Antibacterial Sera
(Compared with Homologous Serum R 12)

Dilution of polysaccharide..................	1/4,000	1/8,000	1/16,000	1/32,000	1/64,000	1/128,000	1/256,000	1/512,000	1/1,024,000
Antidextran R12......................	4	4	4	4	4	4	4	3	1
Antityphoid...........................	4	4	3	2	1	0	0	0	0
Antimorgani...........................	4	4	4	2	1	0	0	0	0
Antisuipestifer........................	4	4	4	3	2	1	0	0	0
Antigallinarum........................	4	4	4	3	0	0	0	0	0
Antiparatyphi A.......................	4	4	3	2	0	0	0	0	0
Antiaertrycke.........................	4	4	4	3	2	0	0	0	0
Anti- R pneumococcus.................	4	4	3	2	0	0	0	0	0
Antipneumococcus I...................	4	3	0	0	0	0	0	0	0
Antipneumococcus II..................	4	4	2	0	0	0	0	0	0
Antipneumococcus III.................	4	3	2	0	0	0	0	0	0
Antienterococcus (1917)...............	4	4	2	2	1	0	0	0	0
Antistrep. ulcer. colitis (1916)..........	4	4	3	2	1	0	0	0	0
Antistrep. ulcer. colitis (1918)..........	3	2	1	0	0	0	0	0	0

Read after 5 hours at 37°C. and overnight in ice box.

More than thirty different antisera were investigated in order to select the ones capable of reacting with the dextran at a dilution of 1–5,000. The results of these tests are shown in Table I.

The dextran was titrated with the reacting sera in various dilutions, using for comparison the specific antidextran serum. The results of this test are shown in Table II. It is of interest to notice the approximate constancy in the titration of the majority of the antisera, the value lying between 32,000 and 64,000. The variation can doubtless

be accounted for by variation in the potency of the sera when tested against their own specific antibody The high titer of the specific antidextran serum is conspicuous.

Having observed the groupings of these antisera into species each group was then studied separately. The first group is the Salmonella and it included *E. typhi*. The results of the titration of the sera, with the dextran at 1–5,000 dilution, are shown in Table III. It is of interest to note that *S. schottmuelleri* (Para B) does not give a reaction with dextran, but this exception can be explained by the fact that this serum does not give specific carbohydrate precipitates. Perhaps the variations with the sera are related to the differences in amount of antidextran antibodies which may parallel those of the specific anticarbohydrate antibodies.

TABLE III

Titration of Antisera of the Salmonella Group and E. typhi with Dextran Polysaccharide (1–5,000)

Antiserum..	Morgani 9	Suipe- stifer 1	Galli- narum 1	Aer- trycke 5	Para A 52028	Para B 2	Typhoid	Control + R 12	Control
1/2	4	4	4	4	4	0	4	4	0
1/4	3	4	3	4	3	0	3	4	0
1/8	1	4	3	4	2	0	2	4	0
1/16	0	4	3	4	2	0	1	2	0

Read after 5 hours at 37°C. and overnight in ice box.

To ascertain whether the antidextran antibody was absorbed by the specific polysaccharide, absorption tests were made with each sera. On one sample the specific carbohydrate was absorbed and on the other the dextran. Each sample was then titrated with the homologous polysaccharide and with the dextran. The results are found in Table IV. No difference is to be observed in any of the absorbed sera, demonstrating that the antidextran antibody is not associated with the specific carbohydrate antibody, but is a definite entity.

The second group studied was the pneumococcus. In Table V the results are given of the titration of different antipneumococcic sera, type-specific and anti-"R" from Types I and II. The type-specific sera gave weaker reactions than the anti-R sera. To ascertain whe-

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ther the precipitate obtained in the type-specific sera was due to the "C" substance, absorption tests were made with the specific and C carbohydrates. The absorbed sera were then precipitated with the dextran, giving the same results as on the control sera that were not absorbed This eliminates the type-specific or the group antibodies (anti-C) as the source of the precipitate with dextran. These results

TABLE IV

Results of Precipitin Test with Homologous Polysaccharide (1–1,000) and Dextran on Sera Absorbed with Same

Test...	Titration of sera with homologous polysaccharide 1-1,000					Titration of sera after absorption with homologous SSS with its own polysaccharide 1-1,000					Titration of sera with dextran 1-5,000 after being absorbed with homologous polysaccharide				
Serum...	*Morgani*	*Suipestifer*	*Gallinarum*	*Aertrycke*	*Para A*	*Morgani*	*Suipestifer*	*Gallinarum*	*Aertrycke*	*Para A*	*Morgani*	*Suipestifer*	*Gallinarum*	*Aertrycke*	*Para A*
1/2	3	4	3	4	3	—	—	—	—	—	4	4	4	4	4
1/4	3	4	3	4	2	0	0	0	0	0	4	4	4	4	3
1/8	1	3	0	4	0	0	0	0	0	0	3	4	3	4	0
1/16	0	0	0	2	0	0	0	0	0	0	1	2	0	3	0

Test...	Titration of sera with dextran 1-5,000					Titration of sera with homologous polysaccharide 1-1,000 after absorption with dextran					Titration of sera with dextran after absorption with dextran				
Serum...	*Morgani*	*Suipestifer*	*Gallinarum*	*Aertrycke*	*Para A*	*Morgani*	*Suipestifer*	*Gallinarum*	*Aertrycke*	*Para A*	*Morgani*	*Suipestifer*	*Gallinarum*	*Aertrycke*	*Para A*
1/2	4	4	4	4	4	—	—	—	—	—	—	—	—	—	—
1/4	3	4	3	4	2	2	4	3	4	2	2	2	3	4	0
1/8	1	4	3	4	2	0	3	2	4	0	0	0	1	2	0
1/16	0	4	3	4	2	0	2	0	3	0	0	0	0	2	0

Read after 5 hours at 37°C. and overnight in ice box.

re in accord with those observed with the Salmonella group as re-ported above.

The third group studied was the *Streptococcus viridans* (Bargen) of different types (Strains 1915, 1916, 1918 and 1838); a strain of *Streptococcus faccalis* (1914), and another of enterococcus (1917). These organisms are closely related. Table VI shows the results of the precipitin test with dextran. It is of great interest to note that

Antisera 1914 and 1916 show a distinct difference in their reaction towards dextran, and as is known they are extremely difficult to differentiate by any other method (except absorption of specific anticarbohydrate antibodies).

All of these strains are immunologically different, their polysaccharides are characteristic, yet while Strains 1916, 1917 and 1918 have

TABLE V

Titration of Antipneumococcus Sera (S and R Types) with Dextran Polysaccharide (1–5,000)

Serum........	Pneumo-coccus I	Pneumo-coccus II	Pneumo-coccus III	Pneumo-coccus R I	Pneumo-coccus R II	Control + R 12	Control
1/2	3	3	3	4	4	4	0
1/4	2	1	1	4	4	4	0
1/8	1	0	0	3	3	4	0
1/16	1	0	0	2	1	2	0

Read after 5 hours at 37°C. and overnight in ice box.

TABLE VI

Titration of Sera from Streptococcus viridans (Bargen) and Similar Organisms, with Dextran Polysaccharide (1–5,000)

Strain No.....	1914	1915	1916	1917	1918	1838
1/2	2	1–	4	4	4	1–
1/4	0	0	4	4	4	0
1/8	0	0	4	4	1	0
1/16	0	0	2	4	0	0

Read after 5 hours at 37°C. and overnight in ice box.

antidextran antibodies, the other three have not. This would appear to suggest fundamental differences in molecular composition of the polysaccharides.

To determine if the antidextran antibody could be absorbed by the specific carbohydrate, Sera 1916, 1917 and 1918 were absorbed with their homologous polysaccharide and then the dextran precipitin test repeated. There was no noticeable difference, this being in agreement with the results obtained with the Salmonella and the pneumococcus groups.

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DISCUSSION

Avery, Heidelberger and Goebel (5) have already described the cross-reactions between the Pneumococcus Type II antiserum and the B. friedlaenderi polysaccharide and vice versa. This crossing they attribute to a similarity in a portion of the complex molecule or a very similar configuration of atoms. Immunological similarity between B. anthrax, meningococci, B. subtilis and B. mesentericus has also been shown (6) and the same explanation offered as that by the above authors.

These experiments suggest that some of the bacterial polysaccharides may contain several active antigenic groups in their molecular structure. Certain of these may be more active in stimulating antibody formation or be acted upon by antibodies of a simpler nature. As immunization progresses, increasing amounts of the complex specific antibodies would be formed in response to the complex bacterial polysaccharide. In a study of the polysaccharides of the Salmonella group their action has been found to be a specific one in agreement with Furth and Landsteiner (7). At the same time it has been possible to show (unpublished work) that the polysaccharides from S. aertrycke and S. suipestifer cross mutually with the respective antisera.

The results in the absorption tests with all the groups studied suggest that the dextran antibody is a distinct entity, for in all cases it remains after absorption of the specific carbohydrate antibodies. It may be that this is a simpler antibody, the primitive or immature stage of the more complex one which is constantly being formed in process of immunization to the bacterial polysaccharides. This work suggests a possible application of the immunological method to the study of the chemical structure of complex carbohydrates.

SUMMARY

Dextran, the synthetic polysaccharide produced by Leuconostoc mesenterioides from saccharose, reacts immunologically with antisera from pneumococci, some of the Salmonella and some of the types of Streptococcus viridans (Bargen).

This immunological relationship is independent of the specific

antipolysaccharide antibodies of these sera, suggesting the existence of a distinct antibody produced by an active group of the specific bacterial polysaccharide, which is similar or identical to the active group of the dextran polysaccharide.

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DETERMINATION OF LUNG VOLUME BY RESPIRATION OF OXYGEN WITHOUT FORCED BREATHING

By JULIUS SENDROY, Jr., Ph.D., ALMA HILLER, Ph.D., and DONALD D. VAN SLYKE, Ph.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

Since 1679 when Borelli (1) first endeavored to measure the air in the lungs interest has attached to the physiological and clinical significance of lung volume determinations. That portion of the pulmonary air which can be expired, either by usual or forced expirations, can be readily measured by spirometers. The residual air which remains in the lungs, however, requires other methods.[1] Of these, two types have proven practical, the nitrogen dilution method and the hydrogen mixture method.

In the nitrogen dilution method, which has been reviewed by Lundsgaard and Van Slyke (2), the subject makes 5 or more forced respirations to and from a bag containing a measured volume of 2 or 3 liters of pure oxygen. Thereby the nitrogen of the pulmonary air is diluted with a known volume of oxygen. From the extent to which the nitrogen content of the gas mixture is diluted below the point, 79.1 per cent, found in ordinary pulmonary air, the volume of nitrogen, and hence of air, in the lungs at the beginning of the experiment can be calculated. Such an experiment must be completed in so short a time, that the difference between the volume of oxygen absorbed in the lungs and the volume of carbon dioxide excreted is not sufficient to affect significantly the total gas volume, and that the accumulation of carbon dioxide does not become great enough to make the gas mixture intolerable to breathe. To achieve mixture of the air in the lungs

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[1] For a discussion of the various fractions into which authors have divided the total lung volume the reader is referred to Lundsgaard and Van Slyke (2). The two main fractions are the "vital capacity," which is the volume of air that can be taken in by a maximal inspiration following a complete expiration, and the "residual air" left in the lungs after a complete expiration. The "middle capacity" is the volume of air held in the lungs at the middle of a normal respiration. For measurement of the "middle capacity" as well as the "residual air" the dilution method or some equivalent must be used.

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with the oxygen in the bag in the permissible 20 or 30 seconds, it is necessary to accomplish the mixture in 5 or 6 respirations. These must approach in size the vital capacity of normal subjects, or mixture will not be complete. The simple dilution method is therefore limited in applicability to subjects who can cooperate, and who can make forced respirations of approximately normal extent.

To overcome these limitations Van Slyke and Binger (3) developed the hydrogen mixture method. The subject respires in a normal manner a mixture of oxygen and hydrogen, in which the volume of hydrogen is known, and the amount of oxygen need not be accurately measured, but is sufficient to maintain respiration for the 5- or 6-minute period found necessary for complete mixture of the gases when the respiration is of only ordinary depth. The carbon dioxide is removed by a scrubber. At the end of the period, oxygen and carbon dioxide in a sample of the respired gas are removed by absorption with alkaline pyrogallate, and the ratio N_2/H_2 in the residual mixture of these two gases is determined by analysis. From this ratio and the known volume of H_2 in the respired gases, the volume of N_2 in the lungs is calculated as

$$\text{Vol. } N_2 = \text{Vol. } H_2 \times \frac{N_2}{H_2},$$

and the volume of air is estimated by dividing this volume of N_2 by 0.791, the proportion of N_2 present in the original pulmonary air.

The hydrogen respiration method can be carried out without cooperation of the subject, and has proved its practicality. Although Van Slyke and Binger used a spirometer, the method can be carried out with only a rubber bag attached to a soda-lime scrubber. The only drawbacks are those necessarily connected with the use of hydrogen for physiological experiments. Every lot of hydrogen must be tested for arsine, since some samples of commercial hydrogen contain this gas in sufficient amount to produce fatal intoxication. Furthermore, one is working with an explosive mixture of hydrogen and oxygen, ignition of which by flame or spark must be guarded against.

To avoid these drawbacks, we have in the present paper devised a technique which permits lung volume determinations without forced breathing, and requires respiration of no extraneous gas other than oxygen. The oxygen is respired from a spirometer, the carbon dioxide being continuously removed by a scrubber. At the end of the period the volume of gas in the spirometer is measured to ascertain the extent to which the pulmonary nitrogen has been diluted. The

calculation is then performed as in the simple nitrogen dilution method. The difference from the latter is that here the oxygen volume, with which the pulmonary air is diluted, is determined by measurement on the spirometer at the end of the respiratory period, instead of being taken as the oxygen volume measured at the beginning. Hence the respiratory period, as in the hydrogen method, can be prolonged as much as may be necessary to obtain mixture of the gases with ordinary respiration.

Since it is difficult to discuss the technique without referring to the factors involved in the calculation, the latter will be considered first.

Calculation

The basic equation of the dilution method (2) is

$$(1) \quad V_L = V_{O_2} \times \frac{N_2}{79.1 - N_2}$$

V_L = volume of air in lungs when the subject is connected to oxygen bag or spirometer; V_{O_2} = volume of oxygen, from bag or spirometer, with which the pulmonary air is diluted; N_2 = per cent of nitrogen found in the respired gas mixture at the end of the period; 79.1 is the average per cent of nitrogen found in pulmonary air by Lundsgaard and Van Slyke (2).

When spirometers of the usual types are employed, the total volume of gas in the spirometer is the sum of the volume indicated on the scale plus the volume in the tubing, connections, scrubber, etc., which is not indicated by the scale. If we indicate the volume shown on the scale by V_S and that held in the dead space as V_D, and substitute their sum for V_{O_2} in Equation 1, we obtain:

$$(2) \quad V_L = (V_S + V_D) \frac{N_2}{79.1 - N_2}$$

The value of the dead space V_D is found in independent experiments with the spirometer used. In place of the lungs a bottle containing a known volume of atmospheric air is substituted and this air is mixed with oxygen in the spirometer. (Or oxygen is placed in the bottle and air in the spirometer.) By a rearrangement of Equation 2, V_D is then calculated, as will be detailed later.

Apparatus

The familiar Roth-Benedict (4) and Krogh apparatuses for determination of basal metabolism can be used without alteration for lung volume by this method. The only disadvantage is the rather

large dead space.   Because of it from 12 to 15 washings with oxygen
are required before each determination to replace the air in the spirom-
eter completely with oxygen.

To obtain an apparatus which has smaller dead space, and which
can be constructed at small cost from ordinary laboratory equipment,
one of the writers (S.) devised that shown in Fig. 1.

Z is a three-way stop-cock, one end of which is connected to the vacuum, the
other to a high-pressure tank of pure oxygen.   The rubber stopper T fits the
rubber mouth piece O.   The aluminum three-way stop-cock S of 20 mm. bore is
made to communicate with the outside air, or through O with the apparatus.
I and E are rubber spirometer valves (inlet and outlet), enclosed in glass jackets.
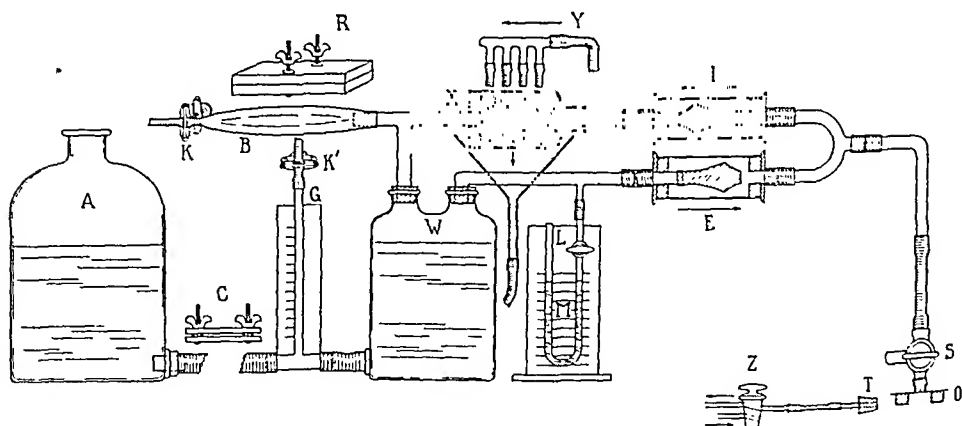X is a 500 to 750 cc. bottle with bottom removed, containing soda-lime.   Copper

FIG. 1. Apparatus for determination of lung volume by the dilution method,
without forced breathing.

gauze of not too fine mesh, or cotton loosely packed, may be used to keep the
particles within the bottle.   Running water enters the cooling system at Y, is
distributed along the sides of the bottle, and runs off into a funnel. B is a rubber
breathing bag, of 5 or 6 liters capacity. R is a press, something like that used for
holding tennis racquets.   It consists of two pieces of wood large enough in area
to cover all but the ends of the bag B. B is also in communication with the
Woulfe bottle W of 8 liters capacity, containing water. G is a tube with a milli-
meter scale.   The bottle W is calibrated on the scale G by pouring in measured
volumes of water, 300 to 400 cc. at a time, so that definite volumes of gas in W
correspond to definite scale readings of the meniscus in G.   The points are plotted
on a curve, from which scale readings can be converted into liters of gas present in
W (1 mm. on the scale of G corresponds to about 30 cc. volume in W).   Reading
G to 0.5 mm. in such an apparatus gives gas volumes in W to $\pm$ 15 cc.   The
flow of water through a suitable length of rubber tubing (of 32 mm. bore) between

A and W is controlled by the clamp C, placed as near as possible to the volume gage G. M is a water-filled manometer with a millimeter scale, connected with the rest of the system through the stop-cock L, which is of at least 2 mm. bore. All of the connecting tubes through which respired air passes are of heavy-walled glass or rubber tubing, of an internal diameter (23 mm.) sufficiently large to avoid resistance to respiration. In order to make the rubber tube connections at the bottom outlets of A and W, it may be necessary to seal a piece of glass tubing into each outlet. This may easily be done by the use of de Khotinsky cement. No temperature control is necessary, since the change in temperature of the gas contained within the system is so small during the period of a determination that it may be neglected. The zero point at the top of scale G is 3 or 4 cm. below the level of the stoppers in W.

Determination of V_D, the Dead Space of the Apparatus

The apparatus is first filled with atmospheric air. If it has contained another gas, it is filled with air and emptied 12 to 15 times to replace other gases entirely with air of atmospheric composition. Then the air content is reduced to that of the dead space, and is mixed with a known volume of oxygen, as follows.

Krogh or Roth Spirometer.—The bell is pressed down until the indicator points to the zero mark on the scale so that no air except that in the dead space remains in the spirometer. Then the mouth piece of the spirometer is connected with a gas container holding a known volume of oxygen. An aspirator bottle like A in Fig. 1 serves as such a container. It is provided at the top with a stopper and a cock by which gas can be admitted and let out, and is connected at the bottom with a similar bottle which is filled with water. A mark is made on the stoppered bottle showing the level at which water stands in it when 5 liters of gas are present. The bottle thus calibrated is first filled to the stopper with water, and then pure oxygen is run in until the water has fallen to the 5-liter mark, the level of the water in the connected bottle being kept even with that in the calibrated one. The calibrated bottle is then connected with the spirometer by a short narrow tube bearing a perforated stopper which fits into the hole in the mouth piece of the spirometer. By raising and lowering the other bottle the oxygen is alternately forced into the spirometer and withdrawn from it 15 times, so that a uniform mixture of the oxygen with the air in the spirometer is obtained. A sample of the mixed gas is then analyzed for nitrogen.

In the above procedure, for economy's sake, oxygen is placed in the bottle at the start, and air in the spirometer, instead of *vice versa*. The calculation of the dead space is similar to that of lung volume in Equation 1.

$$(3) \quad V_D = V_{O_2} \times \frac{N_2 - a}{79.04 - N_2}$$

In this case V_{O_2} represents the volume of oxygen measured into the bottle and then mixed with the air in the spirometer. V_{O_2} is 5 liters when the technique is carried through as above directed. N_2 represents the per cent of N_2 found in the gas mixture by analysis. The per cent of N_2 present as impurity in the oxygen used is represented by a. The N_2 content of atmospheric air is 79.04 per cent.

Sendroy Apparatus.—After the apparatus (Fig. 1) is washed out with air, the bag B is pressed in clamp R and the water in W is raised till the zero mark on G is reached, so that all the air except that in the dead space is removed from the apparatus. A bottle containing a measured volume of pure oxygen is attached to the mouth piece, clamp R is then removed from the bag, and the rest of the determination of V_D is carried out in the same manner as with the spirometers. In this case, however, the volume of oxygen used is 1 instead of 5 liters, and an aspirator bottle of 3 liters capacity is calibrated to hold the oxygen.

Lung Volume Determination

Preliminary Washing of Gases Other than Oxygen Out of Roth or Krogh Spirometer.—Either of these instruments is filled as completely as possible with oxygen and emptied 12 to 15 times to remove all nitrogen. Washing of either of these spirometers requires about 90 liters of oxygen.

Preliminary Washing of Sendroy Apparatus.—W is almost filled with water and clamp C is closed. Then the stopper T is inserted into the hole in the mouth piece. One outlet of the three-way cock Z connects with a suction pump and the other with an oxygen tank. Suction is applied through Z until the bag B is nearly deflated. Strong negative pressure is not applied because it might start leaks in the apparatus. By turning Z, enough oxygen to fill the gas bag is alternately admitted and withdrawn 10 times. The washing can

thus be completed in 2 or 3 minutes, and requires about 40 liters of oxygen.

Addition of Oxygen for the Determination.—With the dead space already filled with pure oxygen, enough more is run into the apparatus to fulfil the requirements of the subject for the duration of the experiment. For a resting subject 3 liters for 5 minutes and 6 liters for 10 provide more than enough.

With the Krogh or Roth apparatus one merely admits 3 or 6 liters of oxygen, measured by the rise of the pointer on the scale, for a 5- or a 10-minute period.

With the Sendroy apparatus the oxygen is admitted as follows. The bag B is flattened by clamping it with R. T is then inserted into the mouth piece S, and enough oxygen is wasted through cock S into the outer air to wash the air out of this cock. Then oxygen is admitted into the apparatus until the water level in W has fallen to a point indicating that the desired amount of gas has been admitted. (This level can be previously determined and indicated by a mark on bottle W.) A reading, V_{S_1}, is made on scale G. Clamp R is then removed from the bag and the latter is filled with gas from W. Clamp C is then closed, and bottle A is left elevated above W.

The Respiration Period.—The determination is carried out in the same way with any of the three types of apparatus.

The subject, with nostrils clamped by a nose piece, is connected to the apparatus by means of the mouth piece, and the cock S is so turned that room air is breathed for several normal respirations. The subject then brings his lungs to the desired position (for residual air he expires as completely as possible), and the cock is turned to connect him with the spirometer or bag. The subject then respires normally for the desired period. 5 minutes are sufficient to obtain a complete mixture for any normal subject. Longer periods may perhaps be needed when the respirations are very shallow. At the end of the period the subject brings his lungs to the same position as at the beginning and the cock S is turned off.

In the Sendroy apparatus it is necessary to admit additional gas into the bag from W at intervals during the period. This is done by opening clamp C and admitting water from A into W. So much must

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In the Sendroy apparatus it is necessary to admit additional gas into the bag from W at intervals during the period. This is done by opening clamp C and admitting water from A into W. So much must

not be admitted at any time that the bag is sufficiently filled to offer resistance to expiration. In particular, when residual air is to be determined, enough space must be left in the bag to receive the final maximal expiration.

Final Gas Measurements and Sampling.—In the Krogh or Roth spirometer the gas volume is read on the scale. The total gas volume in the extrapulmonary part of the system is then calculated by adding the volume, V_{S_2}, indicated by the scale, to the dead space, V_D, previously determined. A sample of gas from the spirometer is then drawn for gas analysis.

With the Sendroy apparatus the following procedure is used. The bag is compressed in the clamp R and the gas is thereby completely driven from the bag into W, as it was when the dead space was determined. The volume V_{S_2} is then read on the scale G. During the reading the cock of M is opened and the bottle A is set at such a level that the water menisci in the two limbs of manometer M are at the same level, indicating exact atmospheric pressure in the system.

To obtain a sample the bag is then refilled with gas from W, and the sample is withdrawn from outlet K of the bag.

Analysis.—The nitrogen in samples of the mixed gases is determined as the residual gas left after absorbing, with alkaline pyrogallol or hyposulfite solution, the oxygen and the small amounts of CO_2 that may have escaped the scrubber. Any suitable gas burette serves for the measurements. A convenient procedure with use of the manometric apparatus of Van Slyke and Neill (5), instead of a gas burette, has been described by Van Slyke and Sendroy (6). It is also especially adapted to determination of the small amounts of nitrogen or hydrogen present as impurity in the oxygen.

Calculation

If the oxygen gas used contains no significant amount of impurity in the form of N_2 or other inert gas, determinable as N_2 by the method of analysis used, the calculation is made by Equation 4.

$$(4) \quad V_L = (V_{S_2} + V_D) \frac{N_2}{79.1 - N_2}$$

If, however, the oxygen used contains amounts of nitrogen which significantly affect the result, the calculation must be made by Equation 5, in which allowance is made for such impurity.

$$(5) \quad V_L = \frac{V_D \, (N_2 - a) + V_{S_2} \, N_2 - V_{S_1} \, a}{79.1 - N_2}$$

In these equations the symbols, V_L, V_D, a, and N_2 have the same significance as in Equations 1, 2, and 3. V_{S_1} represents the volume of gas read on the scale of the spirometer at the beginning of the determination, V_{S_2} the volume of gas read on the scale of the spirometer at the end of the period.

Equation 5 is derived as follows. Using the above symbols, and in addition V_{N_2} to express the total volume of nitrogen in the system of lungs + spirometer, we may calculate the values of V_{N_2} at the beginning and end of the respiratory period, respectively, by means of Equations 6 and 7.

$$(6) \quad V_{N_2} = 0.791 \, V_L + 0.01 \, a \, (V_{S_1} + V_D)$$

$$(7) \quad V_{N_2} = 0.01 \, N_2 \, (V_L + V_{S_2} + V_D)$$

Since the nitrogen volume, V_{N_2}, present in the system is the same at the beginning as at the end of the period, we may equate the right hand members of Equations 6 and 7. Doing so, and solving the resulting equation for V_L, we obtain Equation 5. If $a = 0$, Equation 5 becomes Equation 4.

From the lung volume calculated by either Equation 4 or 5 a small correction is to be deducted for the dead space in the mouth piece used to connect the subject with the spirometer. This dead space is that contained in the tube between the mouth of the subject and the three-way valve, and is usually less than 100 cc. If the space is cylindrical it may be estimated as 0.8 D^2H cc., where D indicates the diameter of the cylindrical section of tubing involved and H is the length of this section, from the mouth end of the tube to the valve. Or this space may be measured simply by closing the valve on the side towards the mouth piece, and ascertaining the volume of water which must be poured in, in order to fill the space.

EXPERIMENTAL

Determinations of lung volumes in the same normal subjects have been performed by the present method, and by the two others discussed in the introduction.

The Dilution Method with Forced Breathing.—The technique of Lundsgaard and Van Slyke (2) was followed. The subject breathed 5 or 6 times in and out of a rubber bag previously washed and filled 3 or 4 times with pure oxygen. The results were calculated according to Equation 1, and are given in Table I.

TABLE I

Determination of Lung Residual Air by the Dilution Method with Forced Breathing

Subject	Residual air
	liters
A	$V_L = 1.42$
	1.54
	1.42
	1.50
B	$V_L = 2.29$
	2.10
	2.34
	2.24

The Present Dilution Method without Forced Breathing.—A preliminary experiment was performed to determine the number of washings necessary with oxygen when the dead space in the Benedict-Roth spirometer was initially filled with air. With a dead space of about 5.9 liters, using about 5 liters for each washing, on the assumption that there is complete mixture of all the gases with each washing, one can calculate the number of washings needed to fill the system with pure oxygen. The result of such a calculation is given in Table II, Column 2. The next column indicates the results actually obtained by analysis after washing with tank O_2 from the breathing end of the apparatus. The oxygen in the tank had been analyzed and found to contain 0.43 per cent N_2. Table II shows that washing is much more efficient than it would be if uniform mixture of each por-

TABLE II

Calculated and Observed Results of Washing. Benedict-Roth Spirometer Apparatus with Oxygen Containing 0.43 Per Cent N_2

| No. of washings | Per cent N_2 | |
	Calculated	Obtained
Beginning	79.05	79.05
1	43.1	8.84
2	43.6	3.75
3	13.0	2.41
4	6.7	2.26
5	3.8	1.31
8	1.0	1.18
12	0.6	0.75
15	0.6	0.8

TABLE III

Determination of Lung Residual Air by the Dilution Method without Forced Breathing. Roth-Benedict Spirometer Used

$$V_D = 5.79 \text{ Liters}$$

| Respiration time | Subject A | Subject B |
min.	liters	liters
1	1.34	1.54
2	1.24 / 1.37	2.15
3	1.41 / 1.42	2.06 / 2.08
4	1.42 / 1.46	2.05 / 2.09 / 1.97
5	1.41	2.17 / 2.00 / 2.07
7.5	1.55	2.09
10	1.58	2.26

tion of oxygen occurred with the gas in the dead space, and that 12 to 15 washings suffice to clear the Roth-Benedict spirometer system of almost all nitrogen except that which is introduced with the tank oxygen. Apparently, when oxygen is run into the spirometer it does not rapidly mix with the gas there, but tends to form a layer by itself and to push the previous gas out.

TABLE IV

Determination of Lung Residual Air by the Dilution Method without Forced Breathing. Authors' Apparatus Used

$$V_D = 0.89 \text{ Liter}$$

Respiration time	Subject A	Subject B
min.	*liters*	*liters*
2		1.80
3	1.45	1.85
4	1.45	2.00
		2.04
5	1.50	1.99
		2.14
		1.93
6	1.45	1.95
		2.06
7	1.50	2.04
8	1.47	2.08
		1.96

In subsequent determinations of V_L with the Benedict-Roth spirometer, the system was washed 15 times with 5-liter portions of oxygen, and the value of 0.75 per cent N_2 was used for a in Equations 3 and 5. Four consecutive determinations of V_D for this system gave values of 5.74, 5.76, 5.83, and 5.78 liters. Table III indicates the results obtained for residual air.

In using the apparatus shown in Fig. 1, it was found that 10 washings of 4 liters each, with the same oxygen used before, reduced the

nitrogen content of the system to 0.54 per cent. This value was used in Equations 3 and 5, for the calculation of V_L determinations by this apparatus. The V_D values obtained were 0.87, 0.89, and 0.89 liter. The V_L values for the same subjects used in Table III are given in Table IV.

The Hydrogen Method without Forced Breathing

The apparatus of Fig. 1 was tested to determine the practicability of its use in connection with the hydrogen method of Van Slyke and Binger. This has the advantage that no V_D determinations are necessary. It was thought that error might arise from loss of hydrogen by diffusion out of the rubber bag, hydrogen, because of its small molecules, being a rapidly diffusing gas. However, actual measurement showed that within the limit of error in reading the scale G, there was no loss of gas over a period of 30 minutes, when the bag and the system were filled with hydrogen.

Three determinations of V_L (residual air) for Subject A were performed. The hydrogen contents of the mixed gases were determined by the combustion method of Van Slyke and Hanke (7). The results, calculated as by Van Slyke and Binger (3), were 1.56, 1.58, and 1.62 liters for 4, 5, and 6 minutes respiration, respectively.

Discussion of Results

The results outlined above indicate good agreement between all of the methods used. Apparently, within the analytical and physiological limits of error, both Subjects A and B were in equilibrium with either the Benedict-Roth or the new apparatus, during the respiration time of 3 to 7 minutes. Subject A was quite remarkably constant over a period of several minutes, by any one of the methods used. That similar results would be obtained with other subjects in all cases cannot be stated. Van Slyke and Binger found that one cannot generalize as to the length of time required to reach equilibrium, since several factors, including the initial volume of oxygen in the spirometer and the depth and rapidity of respirations, are of influence. These authors found, however, that with the hydrogen mixture method

tion of oxygen occurred with the gas in the dead space, and that 12 to
15 washings suffice to clear the Roth-Benedict spirometer system of
almost all nitrogen except that which is introduced with the tank
oxygen. Apparently, when oxygen is run into the spirometer it does
not rapidly mix with the gas there, but tends to form a layer by itself
and to push the previous gas out.

TABLE IV

Determination of Lung Residual Air by the Dilution Method without Forced Breathing.
Authors' Apparatus Used

$V_D = 0.89$ *Liter*

Respiration time	Subject A	Subject B
min.	*liters*	*liters*
2		1.80
3	1.45	1.85
4	1.45	2.00
		2.04
5	1.50	1.99
		2.14
		1.93
6	1.45	1.95
		2.06
7	1.50	2.04
8	1.47	2.08
		1.96

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Dr. Ronald V. Christie has informed us that he has encountered considerable differences in the N_2 content of the pulmonary air of different individuals. A gain in accuracy would therefore be made if this value were determined for each subject, and substituted for 79.1 in the calculation formulae.

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FACTORS AFFECTING THE YIELD OF SPECIFIC ENZYME IN CULTURES OF THE BACILLUS DECOMPOSING THE CAPSULAR POLYSACCHARIDE OF TYPE III PNEUMOCOCCUS

RENÉ DUBOS, Ph.D

(From the Hospital of The Rockefeller Institute for Medical Research)

In a previous paper (1), a method has been described for the isolation from complex organic material of a microorganism capable of decomposing the capsular polysaccharide of Type III Pneumococcus. The principle of this method consists in the use of a selective medium containing as sole source of carbon a small amount of Type III capsular polysaccharide. In this medium, many of the irrelevant bacteria are rapidly eliminated, and only those organisms are selectively stimulated which are potentially capable of decomposing the polysaccharide in question.

Although the medium used had been originally devised for the isolation of the specific organism, it was found that, from cultures of the organism in this medium, an enzyme could be obtained which was capable of decomposing the capsular polysaccharide in the absence of living cells or even cell debris.

Although the synthetic medium—or modifications of it—has lent itself on several occasions to the isolation of organisms adapted to specific purposes, the fact was soon recognized that it was far from being satisfactory for the production of the enzyme. The occasional failure to obtain any active enzyme from cultures of the "S III bacillus" in the original, synthetic medium, led us to investigate the possibility of developing a more dependable technique.

The present paper is concerned with practical methods of production, purification, and concentration of the enzyme, with special regard to the composition of the medium, conditions of incubation of the culture, methods of extraction of the enzyme, and the influence of these factors on the potency and primary toxicity of the preparations.

FACTORS AFFECTING THE YIELD OF SPECIFIC ENZYME IN CULTURES OF THE BACILLUS DECOMPOSING THE CAPSULAR POLYSACCHARIDE OF TYPE III PNEUMOCOCCUS

RENÉ DUBOS, Ph.D

(*From the Hospital of The Rockefeller Institute for Medical Research*)

In a previous paper (1), a method has been described for the isolation from complex organic material of a microorganism capable of decomposing the capsular polysaccharide of Type III Pneumococcus. The principle of this method consists in the use of a selective medium containing as sole source of carbon a small amount of Type III capsular polysaccharide. In this medium, many of the irrelevant bacteria are rapidly eliminated, and only those organisms are selectively stimulated which are potentially capable of decomposing the polysaccharide in question.

Although the medium used had been originally devised for the isolation of the specific organism, it was found that, from cultures of the organism in this medium, an enzyme could be obtained which was capable of decomposing the capsular polysaccharide in the absence of living cells or even cell debris.

Although the synthetic medium—or modifications of it—has lent itself on several occasions to the isolation of organisms adapted to specific purposes, the fact was soon recognized that it was far from being satisfactory for the production of the enzyme. The occasional failure to obtain any active enzyme from cultures of the "S III bacillus" in the original, synthetic medium, led us to investigate the possibility of developing a more dependable technique.

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EXPERIMENTAL

Methods

1. The Organism.—The culture previously described (1) has been carried in the synthetic mineral medium containing the capsular polysaccharide (0.002 per cent) as sole source of energy. For each experiment, the culture is transferred to a medium consisting of a 1 per cent casein hydrolysate (at pH 7.0) in which it grows abundantly. The bacilli are separated from the 18 hour old culture by centrifugalization and washed once in saline before being used for inoculation of the test medium.

2. Medium.—The capsular polysaccharide was prepared from a strain of Type III Pneumococcus by the method previously described (2). The yeast extract used in these experiments is a commercial preparation available under the name "Difco yeast extract." The casein hydrolysate is also a commercial preparation known as "Tryptophane broth."

The soil extract was prepared by heating garden soil with an equal weight of 0.1 per cent sodium carbonate solution for 30 minutes at 15 lbs. pressure.

3. Serological Method for Following the Decomposition of the Specific Polysaccharide.—The presence or absence of the specific substance in the test fluid was determined by the precipitin reaction.

0.5 cc. of the fluid to be tested was added to 0.2 cc. of Type III antiserum[1] and the mixture brought to a volume of 1 cc. by the addition of salt solution. Since the precipitation test gives a positive result with a concentration of specific substance as low as 1:5,000,000, the absence of a positive precipitin reaction was interpreted as evidence of complete decomposition of the specific polysaccharide.

4. Method of Enzyme Titration.—As previously shown (1), there exists a definite quantitative relationship between the amount of specific polysaccharide decomposed and the quantity of enzyme used. The existence of this relation makes it possible to titrate the potency of any given enzyme preparation by the following method.

Varying amounts of the preparation to be tested are added to 1 cc. of a standard solution of Type III capsular polysaccharide (0.001 per cent concentration) adjusted at pH 7.0 with phosphate buffer. The mixtures are made up to 1.5 cc. and incubated for 18 hours at 37°C. in the presence of toluene. They are then tested for the presence of specific polysaccharide by the serological method.

The smallest amount of enzyme capable of completely decomposing the standard amount of capsular substance (0.01 mg.) under these conditions is a measure of the potency of the preparation. It has been found convenient to express the potency of any given preparation in terms of units. A unit of enzyme is defined

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[1] The Type III antipneumococcus serum used in these experiments was obtained through the courtesy of Dr. A. B. Wadsworth, Director of the Division of Laboratories of New York State Department of Health.

as one hundred times the smallest amount which will bring about the complete decomposition of 0.01 mg. of the purified specific capsular polycaccharide in 18 hours at 37°C. The number of units per cc. of preparation is the reciprocal of this smallest amount (expressed in cc.).

For instance, if it takes 0.001 cc. of a certain enzyme preparation to decompose the standard amount of capsular polysaccharide, the potency of the preparation will be $\dfrac{1}{0.001 \times 100} = 10$ units per 1 cc.

It has been reported previously (1) that the S III bacillus grows abundantly on several common bacteriological media such as nutrient broth, peptone solution, casein hydrolysate, etc. The following experiment deals with attempts to determine whether the organism is capable of producing the specific enzyme in media not containing the capsular polysaccharide.

Effect of the Presence or Absence of the Capsular Polysaccharide in the Medium on the Production of the Specific Enzyme by the S III Bacillus

Experiment 1.—Three tubes each containing 5 cc. of casein hydrolysate medium, a fourth tube containing 1 per cent of galactose in 5 cc. of basic mineral medium, and a fifth tube containing 0.005 per cent of capsular polysaccharide in 5 cc. of basic mineral medium, were inoculated with a young culture of the S III bacillus, and the cultures incubated at 37°C. for 48 hours. The cells from two of the cultures in casein hydrolysate were separated by centrifugalization and resuspended in 5 cc. of saline; they were then broken up in one case by repeated freezing and thawing, in the other by extraction at 37°C. for 2 days in the presence of toluene.

The whole cultures and the cell extracts were then titrated for the presence of active enzyme; the results of the titrations are given in Table I.

The results of this experiment indicate that no appreciable amount of specific enzyme was formed when the capsular polysaccharide was not present in the medium. It is worth stating here that the growth was very scant in the specific substance medium although it was fairly abundant in the casein or galactose medium. This shows that the amount of growth is no measure of enzyme production.

The significance of this experiment becomes greater when it is realized that similar tests have been made with over 50 different media including simple and complex saccharides, organic acids, alcohols, pep-

specific substance were simultaneously present in the medium.   Under these conditions there occurred a decomposition of a portion of the capsular polysaccharide by the free enzyme and this decomposition was unrelated to the metabolic activity of the organism.   During the process, inactivation of the enzyme liberated from the autolyzed cells occurred.   The low yields of enzyme may, therefore, be due to these two causes: (*a*) loss of capsular polysaccharide and a resulting decreased amount of growth, (*b*) inactivation of the enzyme already

TABLE II

Effect of the Concentration of Capsular Polysaccharide in the Medium on the Yield of Enzyme from Cultures of the S III Bacillus

Medium containing capsular polysaccharide	Time required for complete decomposition of capsular polysaccharide in the original culture	Titration of enzymatic potency of culture filtrates					
		Specific precipitin reaction given by mixtures of a standard amount of capsular polysaccharide and the following amounts of filtrate of the different cultures (in cc.)					
		0.5	0.2	0.1	0.05	0.02	0.01
per cent	*days*						
0.5	No growth	x	x	x	x	x	x
0.4	No growth	x	x	x	x	x	x
0.3	4	—	—	+	+++	+++	+++
0.2	4	—	—	—	+	+++	+++
0.1	3	—	—	—	—	++	+++
0.05	3	—	—	—	+	+++	+++
0.02	2	—	—	+	+++	+++	+++
0.01	2	—	+	+++	+++	+++	+++

x indicates not done.

Plus signs indicate amount of precipitate formed in Type III antiserum.

Minus signs indicate no precipitation in Type III antiserum (complete decomposition of the specific polysaccharide).

formed.   If such were the case, a possible solution of the problem would be to devise a medium in which the growth would be so rapid that complete decomposition of the polysaccharide would be obtained within 24 hours, while at the same time the rate of autolysis would be decreased so that the release of enzyme into the medium would be retarded.

It is not necessary to report here all the modifications of—and additions to—the synthetic medium which were tested to determine this

effect. Only those changes in procedure will be reported which have given a solution of the problem; *i.e.*, addition of yeast extract to the medium and incubation of the cultures under conditions of increased aeration.

Effect of Yeast Extract on the Yield of Specific Enzyme

Experiment 3.—To three Erlenmeyer flasks (250 cc. capacity) each containing 50 cc. of the basic mineral medium, yeast extract or capsular polysaccharide, or

TABLE III

Effect on the Yield of Enzyme of Addition of Yeast Extract to the Synthetic Medium

Nature of the medium	Time required for complete decomposition of polysaccharide in original culture medium	Titration of enzymatic potency of culture filtrates					
		Specific precipitin reaction given by mixtures of a standard amount of capsular polysaccharide and indicated amounts of filtrate of the different preparations (in cc.)					
		0.5	0.2	0.1	0.05	0.02	0.01
	hrs.						
0.1 per cent yeast extract..........	x	++++	++++	++++	++++	++++	++++
0.1 per cent specific substance.......	72	−	−	++	++++	++++	++++
0.1 per cent yeast extract + 0.1 per cent specific substance..........	24	−	−	−	−	−	+

x indicates no capsular polysaccharide.
Plus signs indicate amount of precipitate formed in Type III antiserum.
Minus signs indicate no precipitation in Type III antiserum (complete decomposition of the specific polysaccharide).

both, were added in the following concentrations respectively: (*a*) 0.1 per cent yeast extract, (*b*) 0.1 per cent specific polysaccharide, (*c*) 0.1 per cent yeast extract + 0.1 per cent specific polysaccharide. The media were inoculated as usual and incubated at 37°C. for 5 days.

The three cultures were separately filtered through Berkefeld filters (N) and the respective filtrates were tested for enzyme action. The readings of the titrations are reported in Table III.

In the culture containing only the capsular polysaccharide in the mineral medium, the specific substrate was decomposed in 3 days,

whereas the same result was obtained within 24 hours in the culture containing both polysaccharide and yeast extract.

Microscopic examinations revealed interesting differences between the three cultures. After 3 days incubation, only long, well formed bacilli were to be seen in the culture containing both capsular polysaccharide and yeast extract, whereas after 24 hours incubation, the other two cultures exhibited only spores and cell debris.

These results as well as the titrations of the filtrates given in Table III clearly show that the addition of 0.1 per cent yeast extract to the synthetic medium increases the rate of decomposition of the capsular polysaccharide by the S III bacillus, retards the process of autolysis, and increases tenfold the yield of specific enzyme from a definite amount of specific substrate. It is worth noting again that, although good growth was obtained in the medium containing the yeast extract alone, no specific enzyme could be detected, confirming once more the results obtained in Experiment 1.

Later experiments have shown that even smaller concentrations of yeast extract serve to bring about the same results. However, the results become less reliable when the concentration of yeast extract is less than 0.03 per cent.

It has been mentioned previously that the S III bacillus is an obligate aerobe. In fact, the aerobic character of this organism is so pronounced that the decomposition of the capsular polysaccharide is much slower when the culture is incubated in a test tube (5 cc. of medium per tube) than when it is exposed in shallow layers in a flask. The following experiment establishes the effect of the conditions of aeration on the rate of decomposition of the specific substance by the S III bacillus, and on the yield of specific enzyme from the cultures.

The Effect of Aeration on the Yield of Specific Enzyme

Experiment 4.—The medium used was the basic mineral medium containing in addition 0.03 per cent yeast extract and 0.1 per cent specific substance. It was distributed in 15 cc. amounts into two Erlenmeyer flasks (300 cc. capacity) and two large test tubes of 3 cm. diameter. This provided conditions of aeration such that the surface area of the culture medium exposed to air was about 50 cm.² in the flasks, and 5 cm.² in the tubes. Flasks and tubes were seeded with a heavy inoculum of a young culture of the S III bacillus.

The specific substance was completely decomposed in 1 day in the flasks and in

2 days in the tubes. On the 3rd day, the culture filtrates were titrated for enzyme activity. The results of the titrations are given in Table IV.

Experiment 4 brings out the very striking facts that (1) as the surface area of the culture medium exposed to air was increased from 5 cm.² to 50 cm.², the rate of decomposition of the capsular polysaccharide was doubled and (2) the yield of specific enzyme in the filtrate was increased tenfold. Although quantitative studies on the amount of growth will not be described here, it can be stated that, roughly speaking, the growth was also ten times heavier in the flasks than in the

TABLE IV

Effect of Aeration on the Yield of Specific Enzyme from Cultures of the S III Bacillus Grown in the Presence of Capsular Polysaccharide

Surface area of the culture medium exposed to air (approximate)	Time required for complete decomposition of the polysaccharide in the original culture	Titration of enzymatic potency of culture filtrates				
		Specific precipitin reaction given by mixtures of a standard amount of capsular polysaccharide and the indicated amount of culture filtrate (in cc.)				
		0.01	0.05	0.03	0.01	0.005
cm.²	hrs.					
50	24	—	—	—	+	+++
50	24	—	—	—	—	+++
5	48	+	+++	+++	+++	+++
5	48	+	++	+++	+++	+++

Plus signs indicate amount of precipitate formed in Type III antiserum. *Minus* signs indicate no precipitation in Type III antiserum (complete decomposition of the specific polysaccharide).

tubes. The emphasis to be placed on incubation of the cultures in shallow layers becomes still greater when one realizes that the conditions obtaining in the tubes would have been considered sufficiently aerobic for most common organisms, since the depth of culture fluid was less than 4 cm. and the medium itself did not have any appreciable reducing power.

The S III bacillus was originally isolated from a mixed culture growing at 37°C. However, the organism can also decompose the capsular polysaccharide at lower temperatures. The purpose of the following experiment was to establish the effect of incubation temperature on the

rate of decomposition of the specific substrate and on the yield of specific enzyme.

Effect of Temperature of Incubation on the Rate of Decomposition of the Capsular Polysaccharide and on the Yield of Specific Enzyme from the Culture

Experiment 5.—The medium used contained 0.1 per cent yeast extract and 0.1 per cent specific polysaccharide. It was distributed in 100 cc. amounts into three Blake bottles which, after inoculation with a young culture of the S III bacillus, were incubated in the horizontal position so as to insure suitable conditions of

TABLE V

Effect of Temperature of Incubation on the Rate of Decomposition of Capsular Polysaccharide by the S III Bacillus and on the Yield of Specific Enzyme

Incubation temperature	Time required for complete decomposition of the capsular polysaccharide in the original culture medium	Titration of enzymatic potency of culture filtrates			
		Specific precipitin test of mixtures of standard amount of capsular polysaccharide and indicated amounts of culture filtrate (in cc.)			
		0.05	0.02	0.01	0.005
	hrs.				
37°C.............	24	—	—	+	++++
30°C.............	48	—	—	—	+
Room temperature.........	120	—	++	++++	++++

Plus signs indicate amount of precipitate formed in Type III antiserum.

Minus signs indicate no precipitation in Type III antiserum (complete decomposition of the specific polysaccharide).

aeration. The temperatures of incubation were respectively 37°C., 30°C., and room temperature. After 2 weeks incubation,[3] the cultures were filtered through Berkefeld filters (N) and the filtrates titrated for enzymatic activity (Table V).

It appears from the results (Table V) that, although the organisms grow more rapidly at 37°C. than at 30°C. the yield of enzyme is higher at the lower temperature. Room temperature is unfavorable, both from the point of view of rate of decomposition and yield of specific enzyme.

[3] The longer period of incubation in this experiment was necessary for the completion of autolysis in the culture at room temperature.

Effect of Length of Incubation and Filtration of the Culture on the Yield of Enzyme

It has been repeatedly mentioned that, as the result of autolysis, the specific enzyme is rapidly released into the medium. However, experience has shown that the greatest yield of enzyme is not obtained immediately after complete decomposition of the capsular polysaccharide, but a few days later. This point is now the object of further analysis and will not be discussed here. In routine procedure, the cultures are incubated for 7 to 14 days before filtration is carried out.

It is of obvious advantage to have the enzyme preparations as clear and cell-free as possible. For this purpose, the autolysates are filtered through Berkefeld candles (N) before being tested. Titrations of enzyme potency of the preparations before and after filtration have always shown that there is no loss of activity during the process. This indicates that the enzyme is not adsorbed on the candle, at least not at the pH (7.0) at which filtration is carried out.

Concentration of the Enzyme Preparations

With the routine procedure now in use in this laboratory, the culture filtrate has an enzymatic activity which varies from 1 to 1.2 units per cc. Attempts were made to concentrate the preparations by the following methods: (a) specific adsorption, (b) precipitation with alcohol and acetone, (c) distillation under reduced pressure, (d) ultrafiltration.

The specific enzyme can be adsorbed with small amounts of kaolin, alumina gels, and bone charcoal, provided the adsorption experiments are carried out at alkaline pH. Unfortunately it has not yet been possible to recover the enzyme from the adsorbent and consequently this method has not as yet proved serviceable.

Precipitation with alcohol and acetone has been only partly successful since it is accompanied by the rapid inactivation of the enzyme (at room temperature at least).

Good results have been obtained by concentration under reduced pressure. During vacuum distillation, the water bath was maintained at a temperature below 35°C., and the vacuum was obtained with an oil pump. Under these conditions, the preparations could be con-

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Incubation temperature	Time required for complete decomposition of the capsular polysaccharide in the original culture medium	Titration of enzymatic potency of culture filtrates			
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		0.05	0.02	0.01	0.005
	hrs.				
37°C............	24	—	—	+	++++
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Good results have been obtained by concentration under reduced pressure. During vacuum distillation, the water bath was maintained at a temperature below 35°C., and the vacuum was obtained with an oil pump. Under these conditions, the preparations could be con-

centrated tenfold without any inactivation of the enzyme. The whole activity could be quantitatively recovered in the concentrate.

Equally successful results have been obtained by ultrafiltration; the optimum formula for the preparation of the membrane on alundum thimbles is as follows:

	gm.
Soluble cotton	6
Glacial acetic acid	122
Anhydride potassium carbonate	2

With this membrane, the enzyme is retained in the ultrafilter and the filtrate is completely inactive. The ultrafiltration method has been adopted in preference to vacuum distillation. When properly carried out, it requires less care and has the special advantage of eliminating in the filtrate a considerable amount of the irrelevant material.

Effect of the Methods of Preparation of the Enzyme on Primary Toxicity

The protective action of the specific enzyme on Type III pneumococcus infections in mice has been described in a previous paper (3). At that time, no primary toxicity of the preparations was observed in the treated animals. Up to 1.5 cc. of the enzyme preparations were injected intraperitoneally into normal mice without any apparent discomfort to the animals. The enzyme preparations then used had been prepared by growing the S III bacillus in the mineral medium containing the capsular polysaccharide as sole source of carbon.

Following the use of yeast extract in the medium, it was observed that the intraperitoneal injection of the enzyme into mice resulted in peritoneal irritation. The same preparation, when injected intravenously into normal rabbits, also exhibited marked toxicity—sudden rise in temperature—and at times death within 2 to 3 minutes after injection.

Two factors have been found to have a profound influence on the development of primary toxicity. Although yeast extract per se is not toxic, cultures in a medium containing this substance prove to be toxic. As a result of this observation, the amount of yeast extract in

the medium has been reduced to the lowest possible concentration compatible with good yields of enzyme; as already mentioned, this minimum concentration is 0.03 per cent.

On several occasions, toluene had been added to the culture medium after growth had developed so as to obtain a more complete lysis of the cells and more rapid release of the enzyme. All the preparations thus treated were highly toxic, even though every precaution had been taken to remove as completely as possible all traces of toluene before use in animals. It seems likely that toluene causes solution of some toxic cellular products which are otherwise retained by the Berkefeld filter.

Purification of the Enzyme Preparations

When the enzyme preparations are obtained by the routine technique now in use in this laboratory, the primary toxicity for normal rabbits is so low that it can be detected only when large amounts are injected. However, attempts were made to further decrease the toxicity by some method of purification.

Dialysis failed since both the enzyme and the toxic principles remain in the dialysate. Specific absorption of the enzyme could not be used since, as already mentioned, the active enzyme could not be recovered from the adsorbent.

A partial solution of the problem was found in devising conditions under which some of the toxic principles would be adsorbed and the active enzyme itself remain in solution. These conditions are fulfilled when a proper amount of aluminum gel (Willstätter's Preparation C) is added to the enzyme previously adjusted to pH 5.5. After a few minutes contact, the supernatant is separated from the gel by centrifugalization. The injection into normal rabbits of this supernatant, in which the specific enzyme is still present, does not affect the temperature and behavior of the animals any more than does a similar injection of normal saline. This technique has always given satisfactory results even when carried out on those preparations which were originally the most toxic. Further studies on the use of these purified preparations in rabbits are presented in the accompanying paper (4).

DISCUSSION

As already stated, the object of the studies described in this paper was of a purely practical nature; *i.e.*, the development of a reliable technique for the preparation of a potent, purified enzyme, capable of decomposing the capsular polysaccharide of Type III Pneumococcus. However, the results have also brought out a few points of interest in the understanding of enzyme production by microorganisms in general.

First, it must be appreciated that the final yield of active enzyme may be no expression of the total amount of enzyme produced by the culture. Several cases have been described in this paper in which both the living microorganism and the enzyme already free in the medium compete for the specific substrate. It appears that conditions may prevail such that the specific substrate may be decomposed by the free enzyme without the microorganism benefiting thereby. This point must be considered in quantitative studies of enzyme production, especially when, as in this instance, the enzyme is quantitatively inactivated in the course of decomposition of the specific substrate.

The high yields of specific enzyme obtained by the addition of small amounts of yeast extract to the medium and by incubation in shallow layers have been traced to a more rapid decomposition of the capsular polysaccharide by the S III bacillus, and a decreased rate of autolysis. Such factors prevent the enzyme released into the medium from expending itself—as it were—on the capsular polysaccharide, thus leaving a larger part of the specific substance as utilizable source of energy for the growing microorganism. However, some more direct form of activation of enzyme production by the yeast extract and oxygen from the air cannot as yet be ruled out.

One cannot help being impressed by the tremendous stimulation of growth in all kinds of media under conditions of increased aeration. This stimulation always expresses itself in the synthesis of a much larger amount of bacterial protoplasm. In media containing the Type III capsular polysaccharide, increased aeration also results in a more rapid decomposition of this polysaccharide, and in larger yield of specific enzyme (Experiment 4).

Finally—and not least interesting—is the fact that, under the con-

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ditions described in Experiment 1, the presence of specific enzyme could be detected only when the medium contained the capsular polysaccharide itself, or the aldobionic acid derived from it. Suggestions of a similar nature are often found in the microbiological literature, but they are the object of much controversy. The case described here seems the more significant since, as already mentioned in a previous paper, the capsular polysaccharide is not an especially favorable source of energy for the S III bacillus, but is attacked only when no other available nutrient is present in the medium. Teleologically speaking, the secretion of the specific enzyme appears then as an emergency measure on the part of the bacterial cell otherwise deprived of growth energy.

SUMMARY

An improved method is described for the preparation, concentration, and purification of a bacterial enzyme capable of decomposing the capsular polysaccharide of Type III Pneumococcus.

The cultural conditions for the growth of the specific microorganism must be such that the capsular polysaccharide is completely decomposed before any appreciable amount of free enzyme is released into the medium. This reduces to a minimum the decomposition of the specific substrate by the free enzyme. As a result, a larger part of the specific substance remains as a source of energy for the growing microorganism and less enzyme is lost through inactivation during the course of decomposition of the specific substrate.

A marked stimulation of growth and of enzyme production occurs when small amounts of yeast extract are added to the medium and when the cultures are incubated under conditions of increased aeration.

Special emphasis is placed upon the fact that, thus far, appreciable amounts of the specific enzyme have been obtained only when the capsular polysaccharide itself, or the aldobionic acid derived from it, was present in the culture medium.

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THE ACTION OF A SPECIFIC ENZYME UPON THE DERMAL INFECTION OF RABBITS WITH TYPE III PNEUMOCOCCUS

By KENNETH GOODNER, Ph.D., RENÉ DUBOS, Ph.D., AND OSWALD T. AVERY, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research*)

Avery and Dubos (1–4) have described an enzyme of bacterial origin which possesses the property of specifically decomposing the capsular polysaccharide of Pneumococcus Type III. When injected together with the infecting organisms this enzyme protects mice against infection with Type III pneumococci and has a curative action when given after a generalized infection has been established.

The present paper deals with the curative action of this specific enzyme in the experimental disease brought about by infecting rabbits intradermally with a strain of Type III Pneumococcus of high virulence for these animals.

The characteristics of this experimental disease in rabbits have been previously described, especially with reference to Type I pneumococcus infections (5–8). The disease offers peculiar advantages for the study of the curative action of a specific agent since the lesion is visible and the course of the infection can easily be followed.

In order to produce the experimental disease with Type III pneumococci it is necessary to use a strain which is highly virulent for rabbits. In these experiments we have used a culture of Type III Pneumococcus designated "PH," a virulent strain previously described by Tillett (9) in his studies of the infectivity of Type III Pneumococcus for rabbits.

The usual strains of Type III Pneumococcus, even when freshly isolated from cases of lobar pneumonia, are not highly virulent for rabbits, and in most instances the symptoms are mild as compared with those resulting from infection with Type I pneumococci. If a sufficient number of organisms are used a fatal infection

can be brought about with some strains but the results are somewhat irregular. Similar experiences have also been reported by Tillett (9), and by Watson and Cooper (10). Occasionally, however, a strain is encountered which is highly virulent for rabbits. Such a strain was employed in these experiments.

EXPERIMENTAL

The methods of infecting the rabbits and of following the course of the disease differed in no essential from those described in previous studies.

Pneumococcus Cultures.—The Type III Pneumococcus used in these experiments was the rabbit-virulent strain described by Tillett (9) under the designation "PH." This was grown in rabbit blood broth and possessed a virulence for mice such that 0.000,000,01 cc. of an 18 hour culture, given intraperitoneally, sufficed to kill within 96 hours. The virulence for rabbits was maintained by frequent animal passage and was such that 0.000,01 cc., given intradermally, caused death or a protracted disease of severe character. With doses of 0.001 cc. only about 5 per cent of untreated animals ultimately recover.

Infection.—Healthy male rabbits of 1,800 to 2,200 gm. were selected, and the hair was removed from the abdominal and flank areas. The animals were then injected intradermally, at a site midway on the flank area, with 0.2 cc. of a dilution of the culture containing the desired number of organisms. In the experiments reported in this paper the infective amount was 0.001 cc. of an 18 hour blood broth culture.

Enzyme Preparations.—The enzyme preparations used in these experiments were, for the larger part, purified and concentrated by the method described by Dubos (4). For the purposes of the present study a unit of enzyme may be defined as one hundred times the smallest amount which will bring about the complete decomposition of 0.01 mg. of the purified specific capsular polysaccharide in 18 hours at 37°C. The exact method for its quantitative estimation has been previously described (4).

Therapeutic Treatment.—The desired amount of enzyme preparation was warmed to 37°C. and injected intravenously at a rate not greater than 1.5 cc. per minute. All preparations were isotonic and were adjusted approximately to pH 7.3.

The development of the lesion and the associated events differ in no essential from those observed in Type I pneumococcus infections, and may be summarized as follows:

After the intradermal injection of 0.001 cc. of an 18 hour culture of rabbit-virulent Type III Pneumococcus in the flank area of the rabbit, there is at first a latent period of 3 to 5 hours. Then, near the point of inoculation, there appear signs of

early inflammation. Edema appears and spreads ventrally at a rate of 2 to 3 cm. an hour, carrying with it the infecting organisms. After 9 to 12 hours the edema has usually reached the ventral midline. More and more edema fluid collects in this area until the entire zone becomes tense and swollen. Within 20 to 28 hours a moderate degree of induration is present and this increases gradually until at 48 hours the involved tissue is quite firm. The lesion is marked by an orange-red color, which in the spreading lesion always develops subsequent to the passage of the edema fluid through a particular area. The local lesion resembles that induced with Type I Pneumococcus except that there is a greater amount of purpura and of frank hemorrhagic necrosis.

With the first developments in the local lesion, the temperature begins to rise and usually reaches a point well over 104°F. at 20 hours, then rises slowly to an average level of 106°F. This high temperature usually persists until the time of death or recovery.

Pneumococci usually appear in the circulating blood 15 to 22 hours after infection and the number rapidly increases. The severity of the disease at any one time appears to be directly related to the number of pneumococci in the blood stream.

Most of the untreated animals die on the 3rd or 4th day of the infection. A few rabbits recover, but only after a course of high temperature persisting for 8 or 9 days.

Course of Events Following the Administration of Enzyme

At the beginning of these studies there existed no basis on which to judge the amounts of enzyme necessary to bring about recovery. It soon became apparent that by the injection of adequate amounts of the specific enzyme 24 hours after the infective inoculation, it was possible to bring about an early and complete cessation of the disease. The blood stream was freed of pneumococci and the organisms disappeared from the local lesion in the course of a few hours. Following the administration of the enzyme, the temperature at first became higher, but fell within 24 hours to normal levels. The local lesion failed to spread and soon showed signs of healing. A detailed account of the findings in treated rabbits follows.

Temperature.—The enzyme has usually been injected 24 hours following the infection, when the rectal temperature is usually 104°F. or higher. Following the administration of the enzyme the temperature rises abruptly, in many instances as high as 107°F. This rise appears to be slightly greater than that occurring after the administration of saline, serum, or vaccines. The peak is generally reached about 2 hours after the injection, but within 4 or 5 hours the temperature begins to

fall and is usually normal within 24 hours.   Provided the amount of enzyme has been sufficient, the temperature remains low.

Blood Cultures.—Pneumococci may be present in the blood stream as early as 15 hours after the intradermal inoculation.   At 24 hours the blood culture is
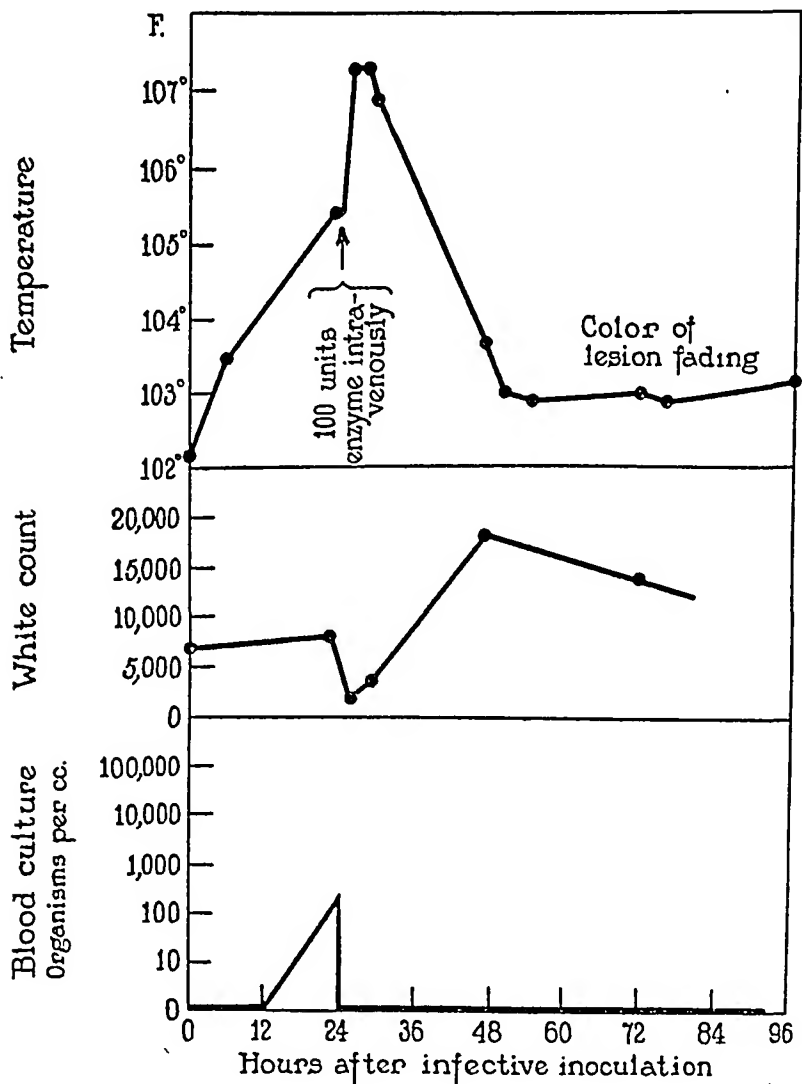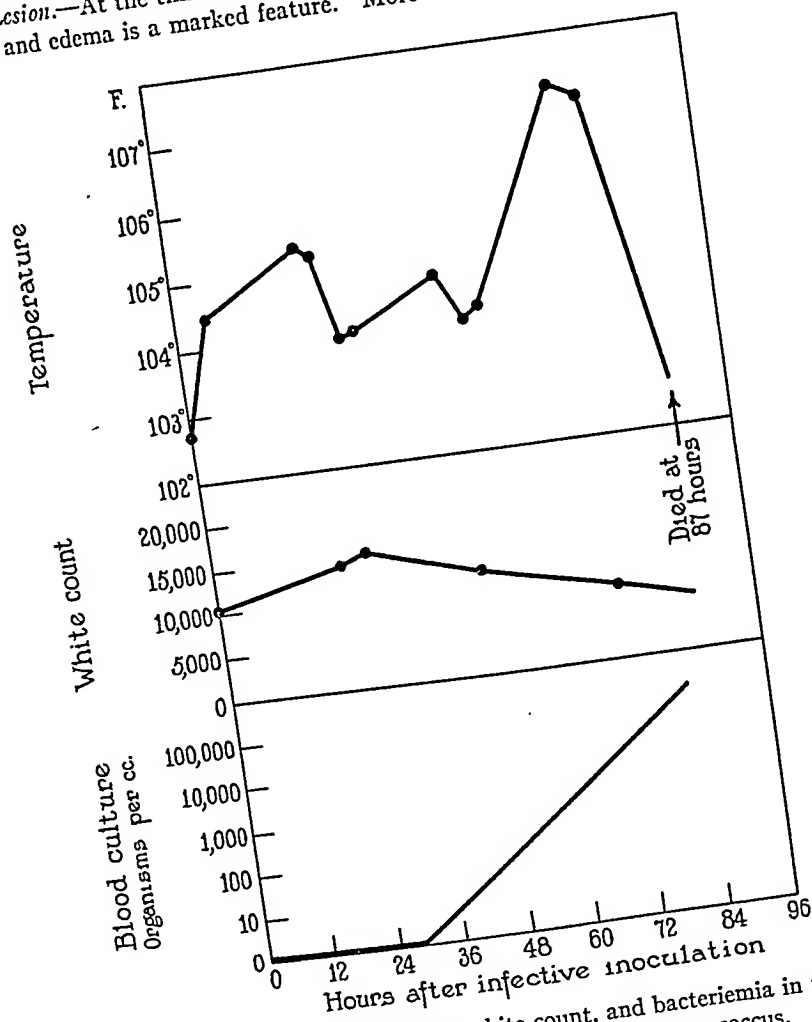

TEXT-FIG. 1.   Chart of temperature, white count, and bacteriemia in the case of a rabbit treated with enzyme 24 hours after infective inoculation with Type III Pneumococcus.

usually positive, the number of organisms per cc. of the circulating blood varying from 5 to 10,000.   Following the administration of suitable doses of the enzyme

the pneumococci promptly disappear from the blood, and there is no recurrence of bacteriemia.

Local Lesion.—At the time of treatment the local lesion shows a bright orange-red color and edema is a marked feature. More or less purpura is frequently in

TEXT-FIG. 2. Chart of temperature, white count, and bacteriemia in the case of a control rabbit infected intradermally with Type III Pneumococcus.

evidence, being more marked in the older and less edematous part of the lesion. The consistency of the lesion is still soft. After intravenous enzyme administration the intensity of the color of the lesion is increased, and in many instances a

large area becomes purpuric within a few hours. With the subsiding of the temperature following treatment the bright color of the lesion begins to fade. After 48 hours all of the non-purpuric areas have again taken on the normal color of the neighboring skin. Following the administration of the enzyme the lesion becomes much firmer and not only does not increase further in extent and magnitude, but actually diminishes. After several days most of the swelling has disappeared. The superficial necrotic material of the purpuric areas is not absorbed but sloughs, and the actual healing of the local lesion is slow and frequently requires many days. With the disappearance of the inflammatory color the surface of the lesion becomes much less glossy, and desquamation, which in other experiments has been associated with healing, makes its appearance.

Direct cultures of material aspirated from the lesion have usually shown viable pneumococci for several hours after treatment, but after 5 to 10 hours the cultures are sterile.

Leukocytes.—Immediately after treatment there is a marked diminution in the number of circulating white blood cells, but within a few hours, usually coincident with the fall of temperature, there occurs a definite increase in the number of these cells, frequently reaching as high as 40,000 per c.mm. The number gradually diminishes and usually reaches normal levels within a few days.

Body Weight.—Weight loss continues as long as the temperature remains above normal, but as soon as the critical changes in the course of the disease occurs, the loss in weight ceases and the original body weight is soon regained.

An illustration of the results obtained in one of a large series of enzyme-treated rabbits is shown in Text-fig. 1. For comparison a typical example of the course and fatal outcome of the infection in the untreated control animals is shown in Text-fig. 2.

The case illustrated by Text-fig. 1 is typical.

24 hours after infection the temperature was 105.4°F. and the blood culture showed 165 pneumococci per cc. of blood. At this time, 100 units of the specific enzyme were injected intravenously. The temperature rose to 107.3°F., but after 5 hours began to fall rapidly. The temperature continued to fall until at 48 hours it was within the normal range. Immediately following the injection of the enzyme the pneumococci disappeared from the blood and did not again make their appearance. The recovery was uneventful.

The control rabbit (Text-fig. 2) had a milder disease than the one which received the enzyme enjection, since 24 hours after infection no bacteria were present in the blood stream. Nevertheless, in the absence of treatment with enzyme, the severity of the disease increased, and the animal died in 87 hours.

The results obtained in the treatment of a large series of infected animals indicate that in cases with severe bacteriemia large quantities

of the enzyme are necessary to obtain successful results, while in animals having fewer organisms in the blood at the time of treatment smaller amounts of the enzyme are adequate.

In our most recent work, nineteen infected rabbits have been treated with large amounts of concentrated enzyme. Of these only one died. On the other hand, among thirty-eight similarly infected rabbits which were untreated, only two survived, and then only after a severe and protracted illness.

TABLE I

Specificity of the Action of the Enzyme

Infections in each case intradermal. Dilutions were arranged so that the required inoculum was in each case contained in 0.2 cc. of broth. Each treated animal received a single injection of 100 units of enzyme (6 cc.) intravenously 24 hours after infective inoculation.

Amount of infective inoculum	Type of Pneumococcus used for infection			
	Type III		Type I	
	Treated	Untreated	Treated	Untreated
cc.				
0.001	S S S	D 88	—	—
0.000,01	—	D 62	—	—
0.000,001	—	—	D 20	D 44
0.000,000,1	—	—	D 23	—
0.000,000,01	—	—	—	D 112

S = survival of animal; in each instance the temperature had fallen to normal levels within 24 hours after treatment and remained within the normal range.

D = death of animal; the numeral indicates the number of hours elapsing before death.

— = not done.

The Specificity of the Curative Action of the Enzyme

In the work of Avery and Dubos, previously referred to, it was shown that Pneumococcus Type III bacteriemia in mice could be checked by the intraperitoneal injection of the specific enzyme and that this action was specific for Type III Pneumococcus. It seemed desirable to investigate the specificity of the action of the enzyme in

this experimental disease in rabbits in which the focus of the infection is more localized and less accessible to therapeutic agents introduced by the intravenous route.

An experiment which illustrates the specificity of the action of the enzyme is summarized in Table I. Both Type III and Type I pneumococcus dermal infections were treated with similar amounts of enzyme, in each instance 24 hours after infective inoculation. Control

TABLE II

Inactivation of Enzyme by Heat

Six rabbits were infected intradermally with 0.001 cc. of rabbit-virulent Pneumococcus Type III broth culture. Two animals received single intravenous injections of enzyme 24 hours after infective inoculation. Two others were given the same amounts of enzyme solution which had been heated at 70°C. for 30 minutes. The remaining two animals similarly infected but untreated served as controls.

Infected rabbits	Enzyme (Lot 2–5–29)	Results
A	Unheated	S
B	Unheated	S
C	Heated at 70°C., for 30 min.	D 38
D	Heated at 70°C., for 30 min.	D 42
E	No enzyme	D 52
F	No enzyme	D 168

S = survival of animal; in each instance treatment was followed by the disappearance of organisms from the blood stream and by a gradual fall of temperature to normal levels.

D = death of animal; the numeral indicates the number of hours elapsing before death.

cases show that the rabbits received at least 100 minimal fatal infective doses of pneumococci in the case of Type III, and ten and 100 minimal fatal infective doses, respectively, in the case of Type I.

In this experiment, of the three rabbits infected with Type III Pneumococcus and subsequently treated with a single injection of the specific enzyme, all recovered, while of the two rabbits infected with Type I Pneumococcus and similarly treated both died. The curative action of the enzyme in this experimental disease, as in the mouse infections, is type-specific.

Heat Inactivation of the Enzyme

It has been shown previously that the activity of the enzyme, as measured by *in vitro* methods and by the protection test in mice, is destroyed by heat. The following experiment was designed to determine whether heating the enzyme also destroyed its curative action in rabbits.

Six rabbits were each infected intradermally with 100 minimal fatal doses (0.001 cc.) of the rabbit-virulent strain of Type III Pneumococcus. After 24 hours two of these animals were treated intravenously with 100 units of enzyme, and two others were given the same amount of enzyme which had been heated at 70°C. for 30 minutes. The two remaining animals were untreated and served as controls. The results of this experiment are summarized in Table II.

In this experiment the animals which received injections of active enzyme recovered promptly. The two rabbits which were given the heated enzyme preparation died, as did also the two control animals. These results demonstrate that the curative principle of the enzyme preparation is heat-labile.

Active Immunity Following Enzyme Treatment

A considerable number of rabbits which have recovered following the administration of enzyme have later been reinfected by intra-dermal injections of pneumococci in order to determine whether or not active immunity had developed. In each case the injection was made in the same area in which the original infective inoculation had been given. The amount injected was 0.2 cc. of an 18 hour blood broth culture of the rabbit-virulent strain of Type III Pneumococcus. This is a massive infective dose, but previous studies (6) have shown this to be the most suitable dose for determining the presence of active immunity. The results obtained in a typical experiment are shown in Table III.

In the animals which had recovered promptly following enzyme injections given 24 to 48 hours after the original infection, no immunity could later be demonstrated. These animals reacted to the subsequent infection exactly like normal animals to an initial infection. On the other hand, in animals which had been originally treated with repeated small doses of enzyme, so that the disease was not arrested

for several days, a high degree of active resistance to subsequent infection was found to be present.

Apparently in the first group of animals the specific antigen was destroyed too early to permit of its functioning as an effective stimulus to antibody formation. These results are analogous to those obtained

TABLE III

Active Immunity in Rabbits Following Recovery from Infection after Enzyme Injections

Each animal reinfected intradermally with 0.2 cc. of rabbit-virulent Type III pneumococcus blood broth culture at the indicated interval after first infection.

Rabbit	Character of original infection	Interval since first infection	Result
		days	
A	Single enzyme injection at 24 hrs., no further bacteriemia	12	D
B	Single enzyme injection at 24 hrs., no further bacteriemia	22	D
C	Single enzyme injection at 24 hrs., no further bacteriemia	27	D
D	Multiple injections of small amounts of enzyme; recurrent bacteriemia for 2 days	27	D
E	Multiple injections of small amounts of enzyme; recurrent bacteriemia for 5 days	27	S
F	Multiple injections of small amounts of enzyme; recurrent bacteriemia for 7 days	34	S
G	Multiple injections of small amounts of enzyme; recurrent bacteriemia for 9 days	34	S
H	Untreated animal which recovered after a long febrile course	22	S
I	Normal rabbit		D

S = survival of animal; localized lesion at point of inoculation; little or no elevation of temperature. High degree of immunity.

D = death of animal; lesion widespread; high temperatures; death at 3 to 5 days. No immunity.

by one of the writers in studying the immunity in animals after recovery following serum treatment (6).

DISCUSSION

These experiments have shown that the enzyme which decomposes the capsular polysaccharide of Type III Pneumococcus has a marked

curative action in the disease brought about by infecting rabbits intradermally with a highly virulent strain of this organism. Following the injection of the enzyme in suitable amounts into the infected animal the blood stream becomes free of bacteria, the focal area of infection becomes sterilized, and the disease process ceases. A mortality rate of 5 per cent in treated cases is in sharp contrast to a mortality rate of 95 per cent in untreated rabbits.

The efficacy of the enzyme in infected rabbits might possibly have been predicted from its protective and curative action in mice. However, the infection in rabbits following intradermal injection of pneumococci is essentially different from the infection which occurs in mice following intraperitoneal injection. In the latter case a generalized infection rapidly occurs. In rabbits, on the other hand, the intradermal injection of pneumococci is followed by a disease which is primarily localized in the skin, and under these conditions the infecting organisms are less accessible to the action of the intravenously administered enzyme.

The present study has not dealt with the mechanism of the action of the enzyme, but in view of the findings of Avery and Dubos with mice, it seems probable that the enzyme brings about the rapid decomposition of the specific capsular polysaccharide, whether it be in the capsules of the bacteria or circulating free in the body fluids. The bacteria freed of their capsules may then be readily taken up and destroyed by the phagocytic cells of the infected animal.

SUMMARY

The action of the enzyme which specifically decomposes the capsular polysaccharide of Type III Pneumococcus has been tested in Type III pneumococcus dermal infections in rabbits. When injected in sufficient amounts, this enzyme is capable of bringing about a favorable and early termination of the experimental disease which ordinarily is fatal in nearly all instances.

The results of the present study yield further evidence that the capsular substance is of great importance in pneumococcus infection, since, in so far as known, the only action of which the specific enzyme is capable is that of decomposing the capsular polysaccharide.

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BRAIN TO BRAIN TRANSMISSION OF THE SUBMAXILLARY GLAND VIRUS IN YOUNG GUINEA PIGS

By N. PAUL HUDSON, M.D., AND FLOYD S. MARKHAM

(From the Department of Hygiene and Bacteriology, The University of Chicago, Chicago)

PLATES 20 AND 21

Peculiar cellular structures in the duct cells of the submaxillary gland of guinea pigs were first noted by Jackson (1) in 1920. Shortly thereafter, these structures were shown by Cole and Kuttner (2) to be nuclear inclusions associated with a virus occurring, apparently, without lethal effect on the host. In this way, a virus of lower animals was discovered, which interestingly demonstrates a natural biological balance between parasite and host. The body responds, however, to the presence of the virus, as pointed out by Kuttner (3) who observed that uninfected guinea pigs are susceptible and infected animals are resistant to the artificial administration of the virus by intracerebral inoculation. Andrewes (4), furthermore, has demonstrated by *in vivo* and tissue culture experiments that immune bodies are in the serum of infected guinea pigs.

That the virus is lethal when cerebrally injected into uninfected guinea pigs is significant as showing that it is not innocuous under artificial conditions and this introduces the possibility that the virus may have natural potentialities other than those of harmless residence in the submaxillary gland. Brain to brain transfer in the guinea pig suggested itself as a possible means to enhance the virulence of the virus so that it might assume under experimental conditions a more important rôle toward its host. Kuttner and Andrewes have independently reported their inability to transmit the virus in this manner with lethal effect. The former author, however, records that transmission was accomplished when massive doses or multiple injections were used and that under such conditions there was only mild mor-

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bidity and no mortality. It seemed to us that the question has sufficient interest to warrant further experimentation and this paper presents the results of our studies on the virus, especially in regard to the successful brain passage in young guinea pigs.

The problem of increasing the virulence of a non-lethal virus has additional interest in connection with the possible significance of cellular inclusions in the salivary glands of man (Ribbert (5), Wilson and Dubois (6), and Farber (7)).

EXPERIMENTAL

The submaxillary glands of six groups of guinea pigs bought in the open market were used as the source of virus. Five of these groups (4 to 6 in each lot) were adult animals, each weighing from 500 to 800 gm. The sixth group was composed of 3 young guinea pigs, 14 days old and averaging 120 gm. in weight.

The age of the animals employed for the intracerebral inoculations ranged from 7 to 32 days. Because of the difficulty in injecting and anesthetizing the very young animals and the danger of encountering spontaneous infections in older ones, we found guinea pigs 2 to 3 weeks old to be the most satisfactory for transmission purposes.

Technic.—The submaxillary glands were removed from the animals of a source group; one gland of each animal was fixed for staining and study, and the others were ground together in a mortar. The emulsion was then centrifuged and a part of the supernatant fluid passed through a Berkefeld N filter. Young guinea pigs were inoculated intracerebrally,[1] each with 0.1 cc. of either the supernatant fluid (hereinafter designated "emulsion") or the filtrate. Brain to brain passage was attempted by injections of 0.1 cc. of emulsion or filtrate of the brain removed aseptically from an animal when moribund or recently dead.

The diluting fluid for tissue emulsions was either sterile water or saline; and the results did not show any advantage of one diluent over the other. The amount of diluting fluid for each gland was 2 or 3 cc., and for each cerebral hemisphere 8 cc.

Bacteriologic examinations, under both aerobic and anaerobic conditions, were made of all gland and brain emulsions and filtrates. Ordinary saprophytic bacteria were grown from the emulsions of two of the six groups of glands, but they were not responsible for death in the cerebrally injected animals in the passage series. This was shown by negative postmortem cultures of the brains. Brain emulsions and all gland and brain filtrates were sterile upon culture.

Glands and brain tissues were fixed in Zenker's fluid and paraffin sections were stained in a variety of ways, principally by Giemsa's method, eosin-methylene blue, and hematoxylin-eosin.

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[1] All cerebral inoculations were made under complete ether anesthesia.

N. PAUL HUDSON AND FLOYD S. MARKHAM

Cell Inclusions in Salivary Glands

The available evidence for the presence of the virus is the finding of cellular changes in the epithelial cells of the ducts. These changes consist essentially in the hypertrophy of the epithelial cell, the appearance of an eosinophilic body within the nucleus, and the frequent occurrence of basophilic strands which bridge the unstained space surrounding the inclusion and extend to the nuclear membrane or to the irregular chromatin masses that lie upon it. In addition, the extranuclear bodies emphasized by Pearson (8) and termed "cytoplasmic inclusions," are often noted (Fig. 7).

Scott and Pruett (9) associate increase in size of the inclusion with the duration of infection. They further conclude that the presence of cytoplasmic inclusions is evidence of a more protracted infection. Our observations on the development of the specific cellular changes in the gland are that: (1) cytoplasmic inclusions occur more frequently in the naturally infected glands than in glands of cerebrally inoculated animals that die; (2) nuclear inclusions appear to be smaller and the affected cells more irregular in the artificially infected guinea pigs; and (3) the nuclear inclusions in adult animals spontaneously infected frequently retain more of the basic dye and seem to be more dense (Figs. 7 and 8).

As the source of virus, the submaxillary glands of 27 guinea pigs, divided into six groups, were removed aseptically and the animals allowed to live. From these six groups, the virus was transmitted in four instances to young guinea pigs by intracerebral inoculation. On the other hand, inclusion bodies were found in the glands of members of only three groups. It appears that the virus is present in the submaxillary glands more frequently than is indicated by inclusion bodies. Serial sections might have disclosed a higher proportion of positive glands. In these experiments, 4 to 10 sections of each gland were examined by the use of a mechanical stage, the fewer number being searched in positive cases. In all, sections from 8 guinea pigs showed cellular inclusions, making about 30 per cent positive. This proportion may be compared with the incidence of 54 per cent found by Jackson, 84 per cent by Cole and Kuttner, and 32 per cent by Andrewes.

bidity and no mortality. It seemed to us that the question has sufficient interest to warrant further experimentation and this paper presents the results of our studies on the virus, especially in regard to the successful brain passage in young guinea pigs.

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It has been stated by various authors that the virus is present in a certain proportion of guinea pigs that have reached the age of 3 weeks or a month. Our sources of virus were adult animals much over a month old, except in one instance when the glands of 3 guinea pigs 14 days old were employed in an attempted control experiment. The virus was successfully transmitted from these young animals and when the sections were prepared, the gland of one was found to be positive. We regard this as a rare instance but of interest in the question of natural incidence of the infection.

The cellular inclusions occurring in the spontaneously infected animals were seen only in the serous portion of the submaxillary gland. We found none in the mucous portion, contrary to the reports of other investigators. The nuclear inclusions were most often in the epithelial cells of the ducts of the magnitude of 10 to 12 cells in circumference. The differing incidence of the inclusions in the positive cases is demonstrated by the observation of a single inclusion in a duct in one case and 133 inclusions in 79 ducts in another, 5 sections being examined in each instance.

Transmission

Serial brain to brain transmissions were attempted in six experiments briefly outlined below. The results of microscopic examination of the source submaxillary glands are presented first in each instance, although actually they were determined after the experiment was under way.

1. Sections of the submaxillary glands of 4 adult guinea pigs showed no typical inclusions. Brain to brain inoculations were fatal to the second transfer, with inclusions demonstrable in the meningeal exudate and the submaxillary glands in the animal of the first brain to brain passage, and in the submaxillary gland of the second fatal transfer.

2. Sections of the submaxillary glands of 4 adult guinea pigs showed no inclusions in the epithelial cells. Cerebral injections were negative. No subinoculations were made.

3. Sections of the submaxillary glands of 4 adult guinea pigs exhibited no inclusions. The 2 animals inoculated intracerebrally with the filtrates died after 17 and 26 days. Brain sections had no meningeal exudate or inclusions, but typical inclusions were found in the submaxillary glands of each. No subinoculations were made.

4. Sections of the submaxillary glands of 6 adult guinea pigs showed very few inclusions in 2 animals. Cerebral injections of gland emulsion and filtrate into 5 guinea pigs resulted in the death of one which had inclusion bodies in the meningeal inflammatory cells and in the submaxillary gland. The attempted brain to brain transfer was unsuccessful, the animal dying the next day.

5. Examination of the sections of the submaxillary glands of 6 adult guinea pigs disclosed affected cells in the ducts of 5 animals. In some cases the cellular involvement was severe. Emulsion and filtrate injections into the brain of 3 young guinea pigs were all fatal, and sections of their brains demonstrated inclusion bodies in the meningeal inflammatory cells. Brain subinoculations from 2 of these animals were lethal in 3 of 6 cerebrally injected guinea pigs, with the inclusions demonstrated in each instance in the cells of the meningeal exudate, and in the submaxillary gland of one animal. The brain emulsion or filtrate from 2 of these animals caused death in 4 of 5 guinea pigs that were cerebrally injected; only one of the 4 had inclusion bodies in cells of the meningeal inflammation and in the submaxillary gland. Further brain to brain inoculations were negative (Table I and Figs. 2–4).

6. Submaxillary gland material from 3 young guinea pigs, 14 days old, was used in an attempted control experiment. Examination of submaxillary sections disclosed a positive gland from one animal. The 3 animals cerebrally inoculated with this material died, and the brain and submaxillary gland of one were positive for inclusion bodies. No subinoculations were attempted.

The above data show that in three experiments brain to brain injections were not done, either because no infection was manifest in the originally inoculated animals or because those fatally inoculated died at night some hours before being found. In the three experiments in which brain to brain inoculations could be attempted, successful passage of the virus was attained in two series. As proof of transmission, we accept the rather rigid requirements of death with the demonstration of nuclear inclusions in a meningeal exudate. This type of evidence is present in one series to the second generation and in the other to the third. Failure of further transmission depended on the same factors as obtained in the negative experiments referred to above. Although gland filtrates, as well as emulsions, were frequently

infective, no proved passage of the virus by filtrates of brain substance was accomplished, suggesting that there may be a quantitative factor in brain to brain inoculations. Apparently, the virulence of the virus was not increased by cerebral passage. Guinea pigs dead 1 or 2 days after inoculation were usually found to have died of cerebral trauma and such results were considered negative. The most pertinent results were in Experiment 5 which is outlined in Table I.

In the three experiments in which transfer of the virus was attempted, there were 23 guinea pigs injected from brain to brain. 10 of these died from 7 to 17 days after inoculation, and meningeal exudate with inclusions in the inflammatory cells was found in 5 of them. On the other hand, 8 of the 10 fatalities manifested cerebral nervous symptoms before death, and a 9th was found dead in the cage without showing symptoms but yielding the specific cell changes in the cerebral sections. It appears that a microscopic meningeal manifestation occurs less frequently than do symptoms and death resulting from the injection of the virus. Furthermore, the virus may be present in a microscopically negative brain, since from such a source it has been fatally transferred to another brain with positive microscopic results.

Findings in the Cerebrally Inoculated Guinea Pigs

Symptoms often appeared 1 or 2 days before the collapse of the inoculated animal and persisted until its death; at other times, however, the acute signs had an early onset and lasted only a few hours. The symptoms were predominately nervous in character. Early evidences of infection in young guinea pigs were muscular weakness and inability to rise when placed on the side. Somewhat later, there were observed local and general tremors spasmodically occurring, escape movements when on the side, opisthotonos, and nervous involvement of the bladder. Terminal symptoms were notably those of exhaustion and marked respiratory difficulty. The interval between inoculation and death ranged from 7 to 17 days.

Significant temperature reactions occurred in some of the animals on the 2nd day after the inoculation. In others, however, when the incubation period was prolonged to 15 days or more, the rise bore no discernible relation to the appearance of the symptoms or the time of

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TABLE I

Experiment 5. Transmission of Submaxillary Gland Virus from Brain to Brain
Source of Virus, Glands of 6 Adult Guinea Pigs

Gland inoculum.........	Emul. No. 34	Filt. No. 35	Filt. No. 36
Guinea pig inoculated...			
Survival or days to death...	7 days	7 days	8 days
Nervous symptoms........	Pos.	Pos.	Pos.
Typical cerebral pathology...	Pos.	Pos.	Pos.

Brain inoculum.........	Emul. No. 38	Emul. No. 39	Filt. No. 40	Emul. No. 41	Emul. No. 42	Filt. No. 43	Emul. No. 44	Emul. No. 45	Filt. No. 46
Guinea pig inoculated...									
Survival or days to death...	Surv.	1 day	Surv.	9 days	15 days	Surv.	Surv.	7 days	Surv.
Nervous symptoms........	—	Neg.	—	Neg.	Pos.	—	—	Pos.	—
Typical cerebral pathology...	—	Neg.	—	Pos.	Pos.	—	—	Pos.	—

Brain inoculum.........	Emul. No. 55	Filt. No. 56	Filt. No. 57	Emul. No. 51	Filt. No. 52
Guinea pig inoculated...					
Survival or days to death...	7 days	12 days	Surv.	17 days	6 days
Nervous symptoms........	Pos.	Pos.	—	Pos.	Neg.
Typical cerebral pathology...	Neg.	Neg.		Pos.	Neg.

Brain inoculum.........	Filt. No. 61	Filt. No. 53	Filt. No. 54
Guinea pig inoculated...			
Survival or days to death...	Surv.	2 days	1 day
Nervous symptoms........	—	Neg.	Neg.
Typical cerebral pathology...		Neg.	Neg.

death.   Thermal elevations generally lasted not more than 24 to 48 hours.

At necropsy, the brains of animals succumbing to intracerebral inoculation with the virus showed no visible meningeal exudate. The dura was sometimes slightly thickened.   Superficial cerebral vessels appeared congested and the cut surface of the brain revealed a mild hyperemia.   Occasionally, there was evidence of hemorrhage in the cerebrum and spinal cord.   No gross pathological changes were observable elsewhere in the body.

Microscopic examination of the brains discloses an irregular involvement of the meninges and superficial cortex, and in some instances of the subependymal tissue.

The changes consist in a mild thickening of the meninges and a variable degree of infiltration with mononuclear cells.   These reacting cells are found in the meningeal spaces as well, and frequently invade the neighboring cortical tissue.   The inflammatory reaction often follows the meninges and their accompanying blood vessels into the upper layers of the brain, where the proximal nervous tissue may be similarly involved (Fig. 1).

The meninges of a particular specimen are not uniformly affected and in negative areas even the perivascular tissue may be normal.   However, where inflammation does occur in the meninges, the regions about the vessels are most intensely infiltrated.   In the upper layers of the cortex, there is a mild perivascular infiltration of some vessels not directly associated with the meningeal folds.   Other small blood vessels, on the other hand, may be entirely normal in this regard, irrespective of their location in the brain substance.

The invading inflammatory cells are mononuclear in structure, only an occasional cell being polymorphonuclear.   The mononuclear cells vary in size, shape, and staining reaction.   Large, weakly stained cells with vesicular nuclei are found together with smaller, more intensely stained cells.   The specific cellular change consisting of nuclear inclusions occurs in a certain proportion of both large and small cells which are commonly irregular in shape.   The inclusions are acidophilic bodies separated from the nuclear membrane by a narrow unstained zone (Figs. 2–4).   A single inclusion occupies a nucleus; it is regular in outline, stains not so intensely as the corresponding structure in the submaxillary gland, and has a uniform appearance.   Occasionally, large cells are seen that have more than one nucleus.   In such a cell, there is sometimes an acidophilic alteration of the various nuclei, similar to the acidophilic change in the mononuclear cells already described.   Phagocytosis is not a conspicuous feature in these sections and has been seen only a few times in the rare areas of hemorrhage and necrosis.   It is noteworthy that cytoplasmic inclusions like those seen in the submaxillary duct epithelium are not

und in the inflammatory cells, and that the essential cells of the nervous tissue ere not found to contain inclusion bodies of either type.

Andrewes records the lesions seen by him in the spinal cord of cerebrally inoculated guinea pigs. We have examined the cord of 5 of the 10 animals dying after cerebral injections and find lesions in each instance. The alterations in the spinal meninges were less severe but resemble those of the cephalic meninges. There is infiltration about the roots of the spinal nerves, and ganglion cells of both the anterior and posterior horns show distinct signs of degeneration. Foci of mononuclear cells and perivascular mantling are frequently observed (Fig. 5).

Recent hemorrhage, though infrequent, is seen in single and multiple areas in both the brain and spinal cord. The hemorrhage varies from a few cells lying in the region of a small vessel to large extravasations associated with necrosis. A few very large mononuclear cells, which may or may not contain inclusion bodies, are found in areas of extensive hemorrhage and occasionally are vacuolated and contain red blood cells.

In one-half of 10 fatally inoculated animals, there was no evidence of inclusions in the salivary glands; all of this negative group died 7 to 9 days after injection. On the other hand, typical nuclear inclusions were found in the glands of the remaining 5 animals, in which 15 days or more elapsed between inoculation and death. In general, the inclusions were smaller and much more numerous in the glands of artificially infected animals than in those spontaneously infected (Figs. 7 and 8). In addition to the characteristic changes in the serous portion of the submaxillary glands of this group, inclusions were also observed in the mucous portion in one instance and in the parotid in three instances (Fig. 6). We did not kill the guinea pigs yielding the source virus and hence the parotid was not examined in spontaneously infected animals; Kuttner observed that the parotid was not involved in the natural infection.

SUMMARY AND CONCLUSIONS

The submaxillary gland virus of guinea pigs was serially transmitted from brain to brain in young guinea pigs. The source of virus was the submaxillary glands of six groups of stock animals. Brain to brain

transfer was effected in two series, in one to the second generation and in the other to the third. The transmission was evidenced by the presence of nervous symptoms and death and by a typical microscopic pathology of the brain. Only certain attempts were successful, ten of twenty-three brain to brain injections being fatal with the specific histopathology present in five. A few observations suggest that the virus may be present spontaneously in the gland and experimentally in the brain without cellular changes being demonstrable, or before they are evident.

While we were able to transmit the virus from brain to brain with fatal results by single injections of small doses, this was not readily accomplished and the transmission failed after two or three passages. We were unable to show any perceptible increase in virulence or adaptation of the virus to the brain tissue of the natural host.

The histopathology was that of a meningoencephalitis. The inflammatory reaction irregularly involved the meninges, the underlying brain substance, and the perivascular tissue of the meninges and upper cortical layer. These structures were infiltrated with mononuclear cells, many of which contained a typical acidophilic inclusion. Congestion of cerebral capillaries uniformly occurred and various degrees of recent hemorrhage were frequently found. Necrosis was noted only when associated with an occasional area of extensive hemorrhage. Similar changes were observed in sections of the spinal cord.

When sufficient time (15 days or more) elapsed between cerebral inoculation and death, typical cellular inclusions were seen in the salivary glands, whereas none was found in animals that died earlier (7 to 9 days). Under the first mentioned conditions, inclusions were demonstrated in the parotid and mucous portion of the submaxillary glands, although in spontaneously infected animals, we failed to find the mucous portion involved and other workers report that the parotid is spared.

About one-third of the stock guinea pigs examined showed cellular inclusions in both the nucleus and cytoplasm of epithelial duct cells of the serous part of the submaxillary gland. From an analysis of the results of brain to brain inoculations, it was evident that spontaneous infection and resistance to cerebral inoculation increased with age. The 3rd week of life is the period of choice for such experimentation.

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EXPLANATION OF PLATES

PLATE 20

FIG. 1. Section of brain of fatally inoculated Guinea Pig 34 (Table I) showing inflammatory reaction in meninges, perivascular tissue, and adjacent cortex. Hematoxylin-eosin. × 150.

FIG. 2. Photomicrograph of meningeal exudate over brain of Guinea Pig 36, showing type of reaction and nuclear inclusion is a large mononuclear cell. Result of primary brain inoculation (Table I). Hematoxylin-eosin. × 1425.

FIG. 3. The same as Fig. 2, for Guinea Pig 45, inoculated with brain of No. 36 (Fig. 2). Hematoxylin-eosin. × 1425.

FIG. 4. The same as Fig. 2, for Guinea Pig 51, inoculated with brain of No. 45 (Fig. 3). Hematoxylin-eosin. × 1425.

### PLATE 21

FIG. 5. Section of spinal cord of Guinea Pig 36, showing focus of mononuclear reaction. Hematoxylin-eosin. × 315.

FIG. 6. Section of parotid gland of Guinea Pig 51; fatal cerebral inoculation (Table I). Nuclear inclusions in epithelial duct cells. Eosin-methylene blue. × 1425.

FIG. 7. Nuclear and cytoplasmic inclusions in a duct cell of the submaxillary gland of an adult guinea pig naturally infected. Eosin-methylene blue. × 1425.
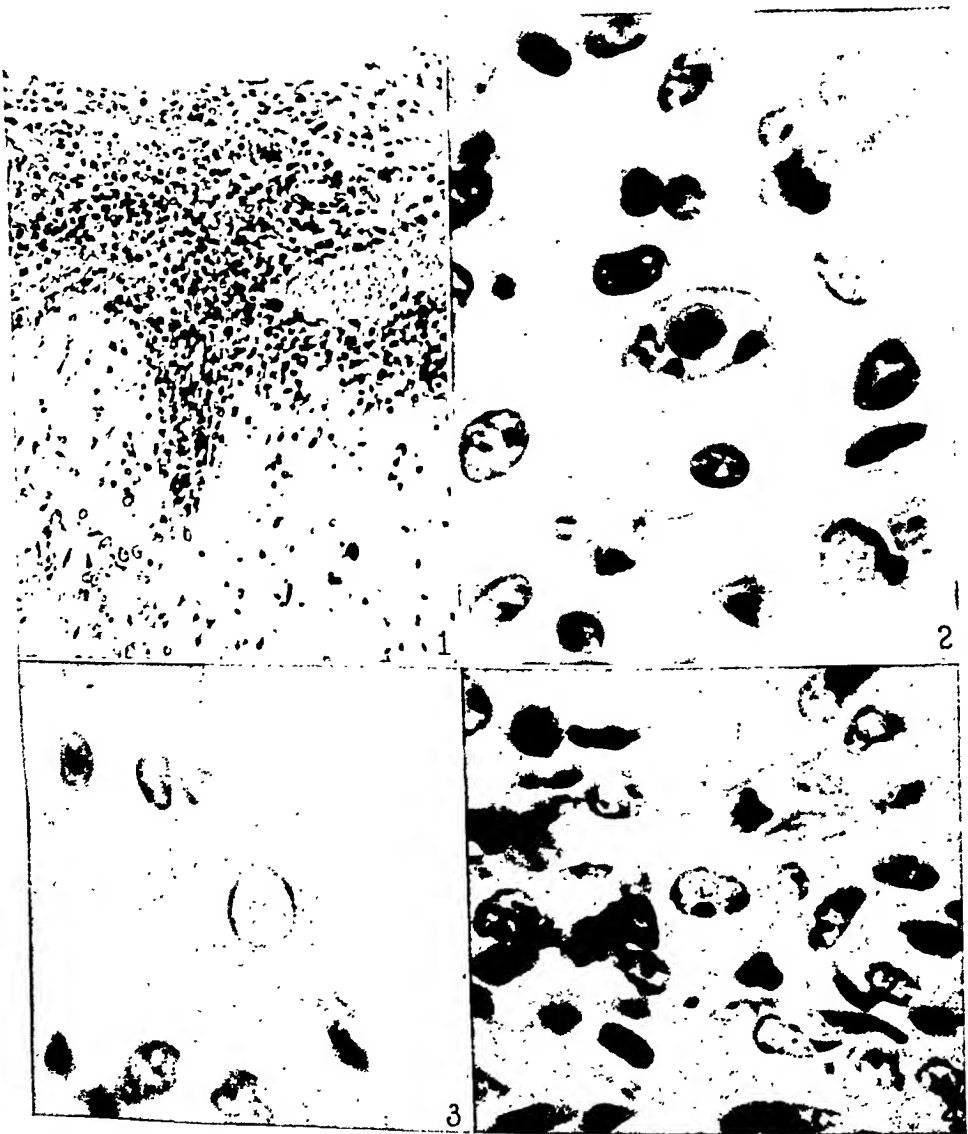
FIG. 8. Section of submaxillary gland of Guinea Pig 51, illustrating small size of nuclear inclusions in cerebrally inoculated animal; compare with Fig. 7. Hematoxylin-eosin. × 1425.
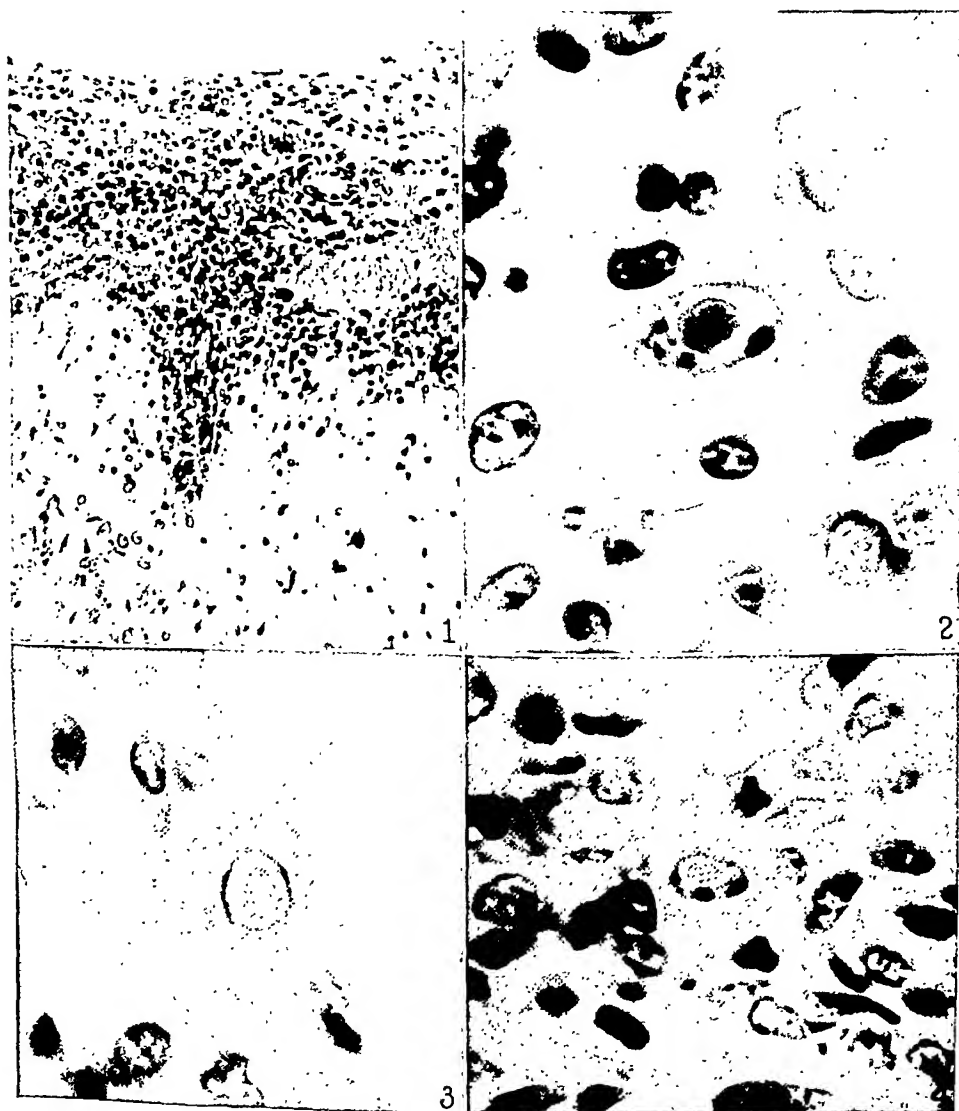
(Hudson and Markham: Submaxillary gland virus in guinea pigs)
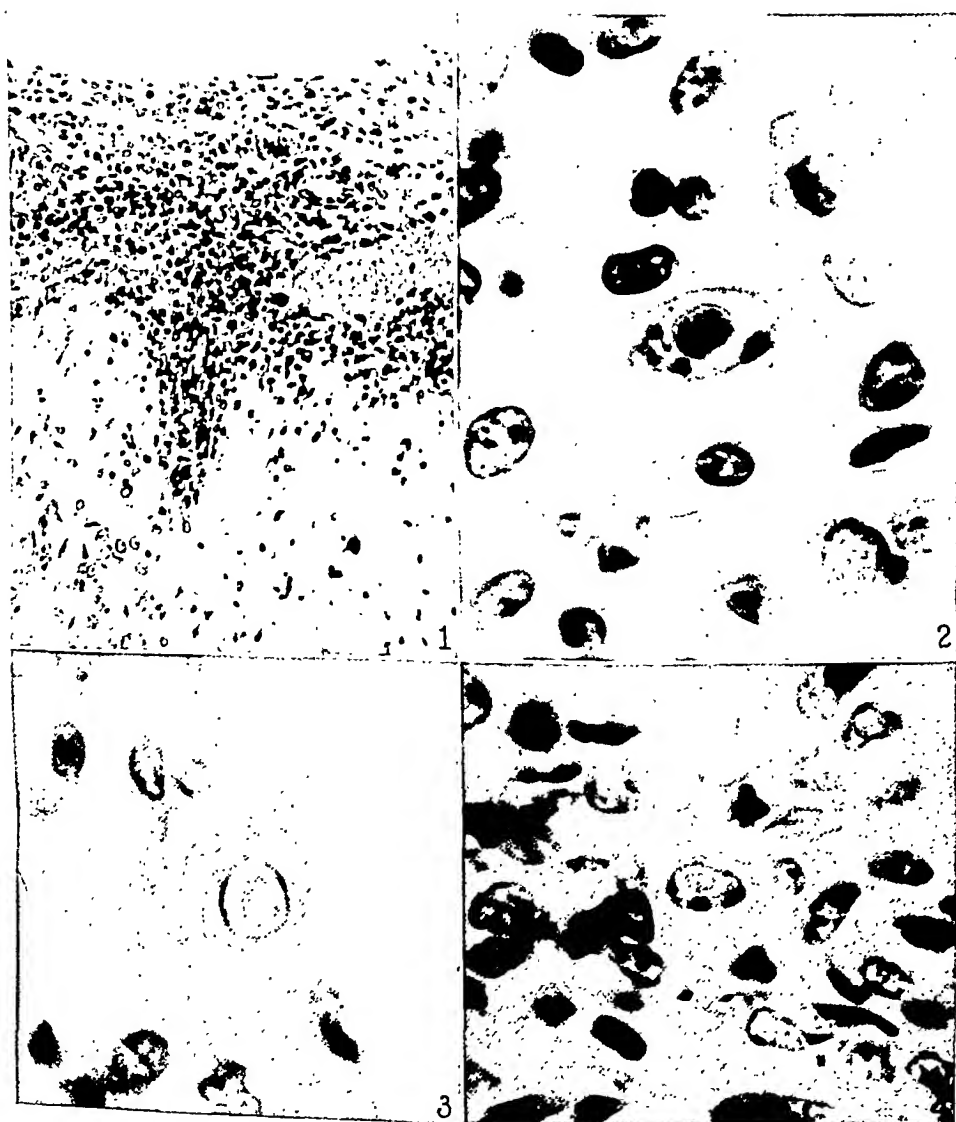
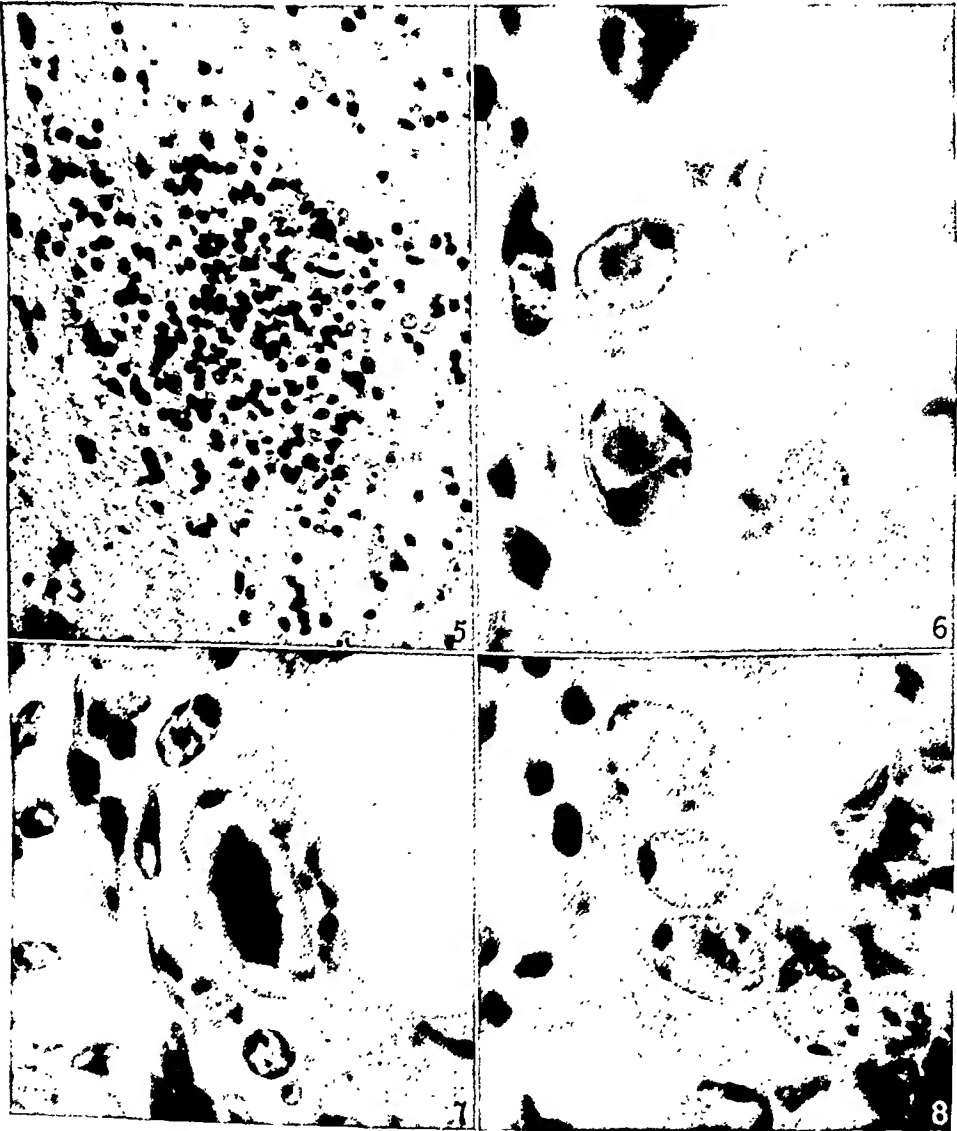(Hudson and Markham: Submaxillary gland virus in guinea pigs)
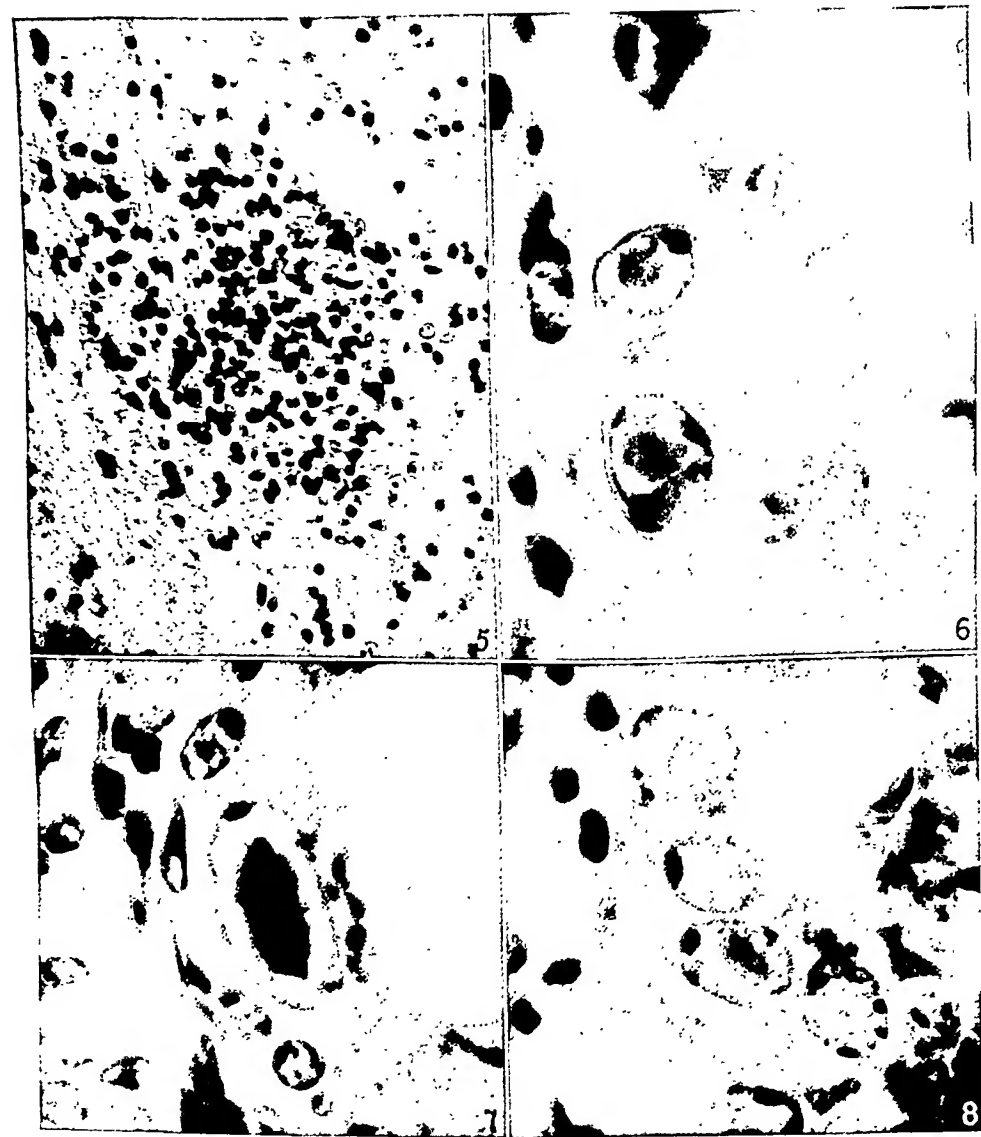
PLATE 20

(Hudson and Markham: Submaxillary gland virus in guinea pigs)

(Hudson and Markham: Submaxillary gland virus in guinea pigs)

(Hudson and Markham: Submaxillary gland virus in guinea pigs)

THE VESSELS INVOLVED IN HYDROSTATIC TRANSUDATION·

By PHILIP D. McMASTER, M.D., and STEPHEN HUDACK, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

The permeability of the walls of the cutaneous venules markedly exceeds that of the capillaries (1). Can it be that under circumstances of pathologically increased venous pressure, as in heart disease or when a limb is constricted, edema develops by a transudation which is localized, primarily at least, to the venules? We have sought to answer this question by testing the influence of slight increases of the venous pressure upon the region and rate of escape of materials from the cutaneous vessels, as indicated by the passage outwards of vital dyes devoid of complicating affinities.

Chicago blue 6B and pontamine sky blue were selected for the tests because the gradient of vascular permeability which importantly conditions the spread from the blood is readily demonstrable with them (2). They pass out of the vessels slowly, but their color is so intense that local differences in the rate of escape are plainly to be discerned. The ear of the mouse was used because the course of events can be followed directly in it; and its veins were obstructed to the desired degree by means of an apparatus developed for the purpose.

Method

The ear was viewed in paraffin oil between parallel glass plates; and before or after the injection of dye into the blood stream the vessels were obstructed near the middle of the organ by means of pressure exerted through a collodion bag.

A rhomboidal platform of white porcelain (1.5 x 2 cm.), fused to the end of a glass rod, is fixed horizontally in one clamp of a Chambers microdissection apparatus, over a cork animal board. On top of the platform, just inside its narrow free edge, is placed a slender, sausage-shaped, collodion bag, about 3 cm. long and 2 mm. in diameter when full of water. The bag connects by a water filled glass

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cannula and rubber tubing with a manometer in which the water has been stained to make the readings easier. A side arm leads to a reservoir and a pump wherewith the manometer column can be raised or lowered very rapidly to any desired level. A looped thread is tied to the free end of the collodion bag and the latter is held in place with pins thrust into the animal board through this loop, and with others set to either side of the cannula (Text-fig. 1).

The mouse, under sodium luminal,—0.2 to 0.23 cc. of a 2 per cent solution for a
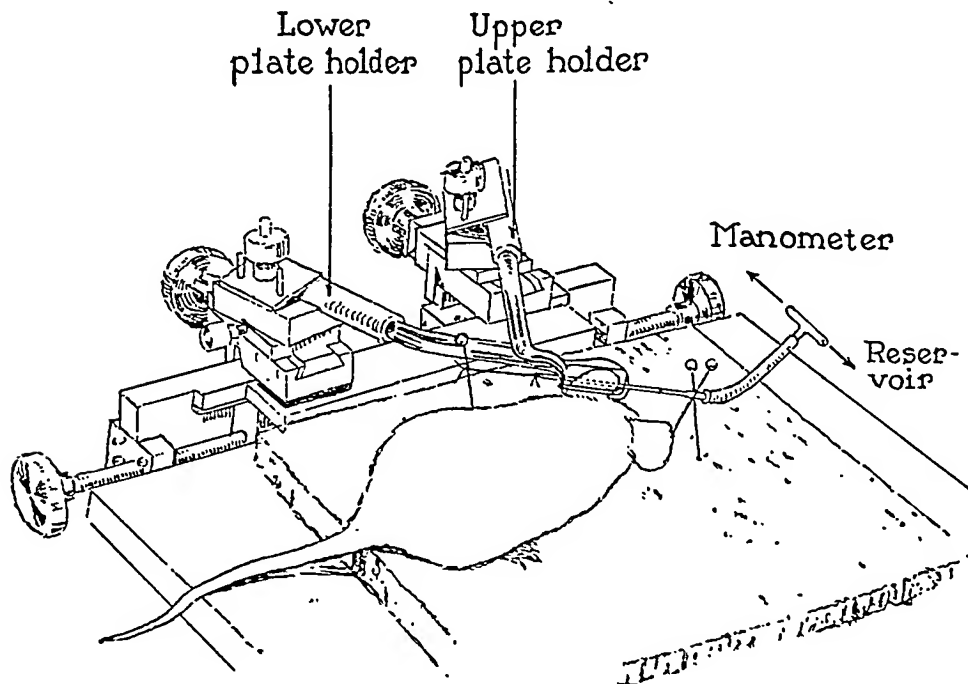
TEXT-FIG. 1. A diagrammatic sketch of the apparatus used for obstructing veins or arteries in the ear of the mouse.

The rhomboidal platform of white porcelain, fused to the end of a glass rod, is placed in the lower plate holder of a Chambers microdissection apparatus. The slender collodion bag stretches across the lower plate and is connected by glass and rubber tubing with the manometer, reservoir, and pump wherewith the manometer column can be raised to any desired level. The ear of the mouse lies on the rhomboidal platform and above the bag. A glass platform similar to the porcelain one is fixed in the upper plate holder of the Chambers apparatus and brought down over the ear.

20 gm. animal, given subcutaneously an hour or two beforehand,—is placed on its belly with the head next the platform, and the latter is so adjusted that the ear rests upon the bag with nearly half projecting beyond. Ear, platform, and bag are now flooded with neutral paraffin oil of low viscosity, and any skin folds are

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smoothed out with a camel's hair brush before another platform, smaller than the first and of transparent glass, is brought down upon the preparation with the aid of the Chambers apparatus. The platforms should be parallel and as far apart as is compatible with compression on filling the bag. The oil renders the hairs invisible when the cooled light from an arc is properly directed by a plane mirror with a universal joint. The blood vessels immediately over the bag, as well as those beyond it, stand out so clearly that both the direct and indirect effects of the pressure changes can be followed with the binocular dissecting microscope. The direct effects are localized to a strip of tissue not more than 1 mm. wide. Only 0.3 cm. of water pressure is necessary to distend the bag, and 1 to 2 cm. causes a visible denting of the walls of the large veins of the ear where pressed upon.

The bags are made out of a 4 to 5 per cent collodion solution in ether and alcohol. Small glass rods coated with caramelized sugar serve as forms; and the sugar is dissolved in 95 per cent alcohol after the bags have dried sufficiently for use. They can be stored in alcohol. When properly made they are flexible, and leak only a negligible amount of water at pressures of 70 to 100 cm.

The bore of the manometer employed is only 0.5 mm.; but since all pressure determinations are relative to a base line determined to some extent by capillarity, this latter factor can be ruled from account. The pressure lag in the bag and the elastic rebound of the rubber connecting tubes can be minimized by avoiding jerky pressure changes.

Comparisons with the free ear of the animal have shown that when the obstructing bag is empty and the platforms at the optimum distance apart for pressure determinations the distribution of dyes is identical in the two. If the bag is close to the head, pressure from it may cause angulation of the cartilage and vessels with untrustworthy results. If it overlies the ear, it sinks irregularly into the soft tissue on distention, stretching and distorting the vessels and often closing some without affecting others of like sort. But when it is on the under side of the organ about half-way to the tip, its pressure is exerted evenly on the cartilage through a thin skin layer, and the ear vessels of the upper side, being pressed between the cartilage and the overlying glass plate, are closed off without evident traction upon them or distortion of the structures to either side. The pressure conditions under such circumstances resemble those with the distensible arm band used clinically. By direct inspection of the vessels one can tell within 1 to 2 cm. how great a column of water is required to shut off veins or arteries.

One worker manipulated the pressure apparatus, a second watched the vascular changes, and a third made notes. Only 2 to 3 seconds were required to bring the column in the manometer to the maximum height required for arterial occlusion, 70 cm., and a much shorter time when lower pressures were worked with.

The Arterial and Venous Pressures in the Mouse Ear

In a preceding paper the vascularization of the mouse ear has been described in detail (1). Its main vessels course upon the outerside

in a radiating fan.   When the apparatus was properly adjusted, all the large veins (or arteries),—which one may liken to the sticks of this fan,—were equally affected by the pressure exerted through the bag.   Repeated readings of the amount required to close off veins and arteries respectively, made at intervals of several minutes yielded identical results of pressure, and so too with determinations upon both ears of the same animal; but when such determinations on the same ear were made one after the other without intermission, they caused a local vasodilatation as shown by the fact that more and more pressure was required to close the veins despite the absence of any arterial pressure rise.   With ether as anesthetic the arterial blood pressure varied almost from moment to moment, being on the average somewhat higher than after luminal.   Pressures sufficient to close off the veins had no effect upon the arterial lumen unless the force of the heart had greatly failed.

Leonard Hill has sought to measure the pressure in the capillaries and venules of the ear of the mouse, applying for the purpose a transparent tambour (3).   The least pressure that sufficed to slow the flow momentarily he took to equal the blood pressure, and using this criterion he concluded that the capillary pressure is only 1 to 3 cm. of water and the venous pressure even less in the bat's wing (4).   But as Krogh has pointed out, what the method really measured was the pressure necessary to disturb the balance between the forces keeping the vessels open as opposed to those which would tend to close them (5).

We have ascertained the amount of pressure necessary to close the veins and arteries momentarily, as shown by the fact that they are emptied of blood in the region directly affected, and have taken this as representative of the maximum pressure within these vessels. Some small part of the force exerted by the bag was doubtless expended in deforming the intervening tissues; but this part is negligible since, as already mentioned, a pressure of 1 to 2 cm. H_2O suffices to dent the veins.   The errors inherent in the indirect method of blood pressure measurement have been often debated.   Fortunately a knowledge of absolute pressures has been unessential to the main purpose of our work, relative ones sufficing.   Nevertheless, the figures obtained provide enlightening information.

In 34 mice of approximately 20 gm. under luminal anesthesia, the pressure required to flatten the fan veins, thus preventing flow, ranged

from 12 to 23 cm. of water. The systolic arterial pressure varied between 33 and 65 cm. Low arterial pressures were associated, in general though not always, with low venous ones, a systolic pressure of 33 cm. with a venous pressure of 12 cm., for example. Mention has already been made of the fact that on stimulation of the ear by rapidly repeated distension of the bag, the venous pressure rose while the arterial did not.

When a pressure was maintained which just sufficed to shut off the veins, flow was resumed within 10 or 15 seconds. And if now the pressure was raised still further, again just producing occlusion, the obstacle was once more overcome. Each successive slight increment was but transiently effective, and the pressure which closed the veins permanently was only a few centimeters less than that which shut off the arteries. For example, in one case the veins were not permanently occluded until 54 cm. H_2O had been reached, while at 56.5 cm. flow in the arteries was intermittent and jerky, ceasing entirely at 59 cm.

To explain the rapid rise of pressure back of a venous obstruction one thinks first of arteriovenous anastomoses such as exist in the extremities of various species (dog, cat, rabbit) (6). Grant has recently provided an excellent study of those of the rabbit ear (7). They are short cross-connections, often contorted, between vessels lying side by side; and normally they are closed. In the mouse ear injected with India ink gelatin mass none has been found (1); and the rapid injection of vital dyes into living mice, while the upper or lower side of the ear is watched, does not disclose any, although the course taken through the vessels by the advancing columns of stained blood can be plainly seen. Long, straight capillaries exist at the very edge of the ear, paralleling its outline, and flow in these is rapid and continuous, but it is no more rapid than in many capillaries toward the middle of the organ. Grant was frequently enabled to discover anastomoses in actively hyperemic rabbit ears by pulsations where the arterial blood directly entered a vein. We have rapidly injected dyes into mice with ear vessels that had become distended as result of almost complete venous occlusion by the pressure bag and have sought to perceive an entrance of stained blood by way of by-passes before the main flow entered the veins. It never occurred.

Always the stained blood took the way of the capillary web. It seems safe to conclude that effective arteriovenous anastomoses do not exist in the ear of the mouse.

When suddenly blocked the veins do not at once widen. The rapid mounting of pressure behind the obstacle presented by the collodion bag might conceivably be due to a fixation of the capillaries in tissue which does not give. But when the ear is touched on its upper surface with a rounded glass point the tissue is seen under a magnifying glass to dimple and to be loose, while furthermore there is room in it for the rapid accumulation of much edema fluid. This happens when a pressure is exerted upon the veins that is sufficiently high to shut them off while the arteries still pump blood in. Only when the obstacle to outflow is so considerable does capillary dilatation become well marked. A pressure which suffices merely to narrow the veins and to interfere to some extent with flow through them, as shown by a more rapid current where the bag indents the vessel, causes, it is true, some capillary widening as can be seen when the vessels distal and proximal to the bag are compared after the blood has been darkened with dye. But this widening is very slight. Krogh has stressed the fact that some of the contracted capillaries, of muscle especially, are not opened by great pressure (5), and Tannenberg and Fischer-Wasels state that after veins have been tied off the capillaries do not at once dilate (8). The present work shows that the patent capillaries of the mouse ear do not immediately give way when subjected to arterial pressure but transmit this with but little loss to the venous blood.

Effect of Increased Venous Pressure on the Escape of Substances from the Blood Stream

The opportunity for vital dyes to escape from the blood into the corium of mouse skin, whether of the ear or of the body, increases along the further portion of the capillary web and is greatest in the region of the primary venules. This is not because of a more favorable ratio of wall surface to vascular content. It results from intrinsic local differences in vascular permeability. During the first minutes after an intravenous injection of Chicago blue 6B bright blue patches develop in the corium of the ear, owing to an escape of dye from the further part of the capillary web and the adjoining venules before

any gets out elsewhere. Eventually the staining becomes uniform owing to a redistribution within the tissues. With the more poorly diffusible pontamine sky blue the patches develop more slowly and are smaller, while with highly diffusible dyes (brom phenol blue, patent blue V) some general coloration develops at the same time as the patching, because the capillary web is everywhere permeable to these dyes, though unequally so. These findings have been illustrated in a preceding paper (1).

In the present experiments a known degree of obstruction to the venous outflow was produced, Chicago blue or pontamine blue was injected, and the ear compared with its fellow. The course of the staining was followed under the microscope in the organ that was pressed upon, differences in the distribution of stain to the tissue proximal and distal to the pressure bag being readily seen. At various times after the injection both ears were lopped off, the pressure was only then relaxed, and the specimens were compared while side by side in oil under a single cover slip with their proximal halves, wherein no difference could be expected, blocked from sight with squares of white paper.

When the systolic pressure was very low, the ear with partially obstructed veins did not stain as well as the control because the arteries were compromised by the pressure exerted, as could be directly observed, and the turnover of stained blood was cut down. All such instances were discarded. In mice with a vigorous circulation (and a venous pressure of 18 to 23 cm.) it was found that a water column 5 to 6 cm. high was required to narrow the veins sufficiently for recognizable interference with outflow. Slighter pressures caused some denting of the veins but the latter were still so broad that there was no visible change in the blood current; and the staining in such instances did not differ from that in the control organ. Pressures of 5 to 6 cm. had pronounced results on the staining. Pontamine blue and Chicago blue began to escape at once from the small venules of the ear and the further portion of the capillary web, with result that the characteristic, patchy staining was already marked at a time when no dye had emerged in the region proximal to the pressure bag and none in the control ear. The subsequent coloration was more intense than in the control tissue. Its distribution in relation to the vessels was not altered from the ordinary, however. With pressures of 12 and 13 cm., which did not prevent an abundant rapid flow through the veins though compressing them to half size or less, the ear again became patched with blue more quickly and intensely than its fellow, and the patches were larger than ordinary, because dye escaped into the tissues further back along the capillary web in the

direction of the arterioles. In addition a narrow zone of deep blue formed along the larger veins, in some cases even along the largest (fan veins), and there was some diffuse staining of the ear as a whole before any developed in the control. In animals having a very low arterial blood pressure, and excluded for such reason, some of these differences in distribution could be noted despite the fact that the staining was less intense than that in the control ear.

The findings show that a slight interference with venous outflow enhances the escape of dye through the walls of the venules and the adjoining portion of the capillary web. When the interference is more considerable these effects extend further back along the capillary web, and veins not ordinarily permeable let dye through. A typical result of such interference has been photographed in Fig. 1. Even when the changes are very marked the color pattern still indicates that the opportunity for escape from the blood is greatest in the region of the primary venules and least in the proximal part of the capillary web, that near the arterioles.

As already mentioned, the smallest pressure increase causing an evident obstruction to venous outflow gave rise also to a perceptible widening of the capillaries. This was unaccompanied by any pronounced increase in their general permeability, a fact sufficiently attested by the unaltered though accentuated staining pattern. With a sustained pressure obstacle of 12 to 13 cm. of water, distention of the capillaries was considerable and edema of the ear developed, as shown by thickening of it, pitting under pressure, and an almost complete emptying of the vessels when the organ was cut off. The ear colored rapidly but the existence of a gradient of distribution was plainly to be perceived.

In an accessory group of experiments the maximal venous obstruction compatible with flow was produced,—that is to say, the pressure in the bag was raised to within a few centimeters of the systolic arterial pressure and maintained for a few minutes. During this period the capillary web became greatly distended and solid columns of red cells formed in some of the meshes. Now dye was injected. It at once passed out everywhere along the capillary web, except from the blocked meshes, and a deep, generalized staining rapidly developed, without trace of the pattern indicative of the ordinary gradient of capillary and venular permeability. Staining of the same sort occurred when

the pressure obstacle had been done away with just prior to introducing the dye, as also when the local circulation was stopped by bag pressure just after the stained blood had been distributed through the vessels. Evidently the distention of the capillaries was accompanied by a great increase in the permeability of their walls, a change which was not immediately reversible.

When an anesthetized mouse is suspended head downwards, the arterial and venous pressures in the ear mount rapidly and the organ becomes engorged with bright blood. We have followed the phenomena with the anesthetized animal hanging by the hind legs over a platform on which the ear is spread as usual. Repeated rapid distention of the collodion bag causes the pressure to rise under ordinary conditions, but it is a far more effective stimulus when the animal hangs head downward. There is a prompt return to ordinary pressures, however, when the mouse is placed once again on its abdomen. These facts can be illustrated by the following protocol.

Mouse weighing 18 gm., given 0.21 cc. of a 2 per cent solution of sodium luminal into the subcutaneous tissue 1½ hours prior to the experiment.

The venous pressure in the ear with the animal on its abdomen was 21 cm. H_2O and the systolic arterial pressure 3 minutes later 40 cm. Now the mouse was hung head downwards for 12½ minutes. The venous pressure at the end of this time proved to be 27 cm. H_2O, with an arterial pressure of 65 cm. and jerky flow at 62 cm. A pressure of 30 cm. exerted 1½ minutes after this last reading failed to shut off the veins and so too did 35 cm. after 45 seconds more, but 40 cm. applied after another minute, occluded them. The ear was now engorged with bright blood.

The animal was replaced on its belly and 4 minutes later a pressure of 20 cm. sufficed to shut off the veins. Repeated readings at short intervals yielded the same result, and the arterial pressure, taken next, proved to be 54 cm. with pulsatile flow at 52 cm. A minute afterwards the venous pressure was between 23 and 25 cm.; and finally, after 2 minutes more, the arterial pressure was once again 54 cm. H_2O.

The distribution of dyes was followed in some of the suspended animals. Controls, of identical weight, anesthetized in the same way but lying on the abdomen, were injected simultaneously. When the animals had been suspended for a few minutes only, the character of the staining showed that the gradient of vascular permeability still existed; but the coloration developed sooner and was much more

intense than in the controls, dye escaping from all of the capillary meshes except those immediately next the arterioles. In addition a zone of stain formed just outside the larger veins. The rapidity with which the dye was carried through the vascular web showed, as had the color of the ear prior to staining, that an active hyperemia was present.

Some mice were suspended for 3 to 4 hours before receiving the dye. The position was well tolerated, but in some cases a slight edema of the ear had developed at the time of injection. At the end of the preliminary period the fan veins had widened markedly, and so too with the lesser veins and venules, the changes persisting for an hour or more after the animals were again prone, as did also the vascular engorgement and high venous pressure. Dye injection while the animal was still suspended caused a rapid, generalized staining, with a broad zone of deeper color along the large fan veins. The fact could still be discerned however, that the staining was progressively more intense along the capillary web and greatest in the tissue about the venules. It was plain that a gradient of permeability still existed along the capillaries, reaching its peak in the venules.

DISCUSSION

Under normal circumstances a mounting gradient of permeability exists along the further portion of the capillaries supplying the corium; but the venules into which they empty are more permeable still (1). The present experiments prove that slight increases in venous pressure increase the opportunity for the passage outwards of dye substances from the venules and the further portion of the capillary web without essentially modifying the conditions elsewhere. Greater increases have the added effect of causing the capillary wall further back toward the arteriole to become unusually permeable. Since there is some attendant dilatation of the capillaries one cannot be certain whether the more abundant escape of dye is due to a graded increase in the amount of surface through which diffusion can occur, with some increase in local permeability due to thinning of the wall, or whether the heightened hydrostatic pressure has caused active filtration. Perhaps all these influences are at work. The wall of the larger veins is certainly rendered more permeable by the pressure, for it lets through materials which ordinarily do not pass. Nevertheless the venules remain the most permeable of all the small vessels. Only when the venous pressure is raised nearly to that in the arteries,

and the capillaries, as result, have been forcibly distended, does the characteristic gradient of vascular permeability disappear.

In mammalian skin, especially that of human beings, venules largely take the place of capillaries; and they are differentiated for special functions (9). In voluntary muscle on the other hand the arrangement of the venules, transverse to the muscle fibres, indicates, like their shape and size, that they are merely drainage channels. In muscle the vascular permeability is greatest toward the end of the capillaries, and it is here that a heightened venous pressure exerts its greatest effect, not in the region of the venules (10).

The dyes employed for the observations do not at once become fixed upon, or stored in, the skin but color it because contained in the intercellular fluid into which they pass from the plasma through the barrier of the vessel wall (10). The point is an important one in the present relation because venous pressures which suffice to extend and emphasize markedly the gradient of vascular permeability give rise to edema at the same time. There can be no doubt that the region of greatest escape, under such circumstances, of dyes dissolved in the plasma will also be that of greatest fluid escape.[1] One is justified in inferring from the color pattern that transudation through the small venules is more important for the rapid development of edema of the skin as result of increased venous pressure than is transudation through the capillaries. Edema occurring as the result of vascular injury by heat or cold, on the other hand, comes about mainly by loss of fluid from the capillaries as shown in an accompanying paper (11). Several explanations of the edema of heart disease have been offered in the past (12–15). Not only are the small vessels caught, so to speak, between the arterial pressure and an abnormally high venous one, but nutritive or toxic disturbances of the vascular endothelium may occur and affect permeability. The endothelium of the venules should suffer as much from these problematic disturbances as that of the capillaries, if not more. Most of the edema fluid accumulates in the subcutaneous tissue. Whether it finds its way there secondarily from

[1] This is not to say that wherever dyes escape from the blood under ordinary circumstances there must be a flow of water as well. They pass out by diffusion, in the absence of hydrostatic pressure, and yield the color pattern indicative of the ordinary gradient of vascular permeability (10).

the skin or is the result of fluid escape from the relatively infrequent subcutaneous vessels is a problem as yet unsolved.

The abnormal permeability of large veins widened by high pressure (as evidenced by the escape of dyes into the ears of mice suspended for long periods head down) needs no comment. One may recall in connection with it the seepage of fluid from the abnormally distended veins of human beings.

The capillaries in the soft tissue of the mouse ear transmit pressure with but little loss, as shown by the rapidity with which this mounts behind an obstacle to venous outflow. Our observations confirm those of Landis (16) who found by direct determinations on human capillaries that temporary decreases in the venous flow (as in Valsalva's experiment) cause a prompt intracapillary rise. As he points out, the capillaries that he punctured for the purpose of pressure readings were supported by the firm tissue of the nail-bed and their walls were relatively rigid. This was not the case in the mouse ear. In the mouse ear the least increase in venous pressure that enhances the permeability of the venules, as shown by the rate of escape of dyes, causes also a perceptible widening of the capillaries; but the gradient of permeability along these latter is almost unaffected. High pressures do away with the gradient completely. The uniformity of the staining that develops when the capillary barrier has been broken down by such pressures attests to the fact that the color pattern developing under ordinary circumstances is not due to structural differences in the tissue surrounding the capillaries. Previous work from this laboratory has ruled out the possibility that it is the result of a graded tonic contraction of these vessels or other functional conditions (10). There is good reason to refer it to a structural differentiation along the capillary.

SUMMARY

The gradient of permeability which exists along the cutaneous capillaries and venules is accentuated and broadened in scope by increasing the venous pressure moderately. Under such circumstances transudation leading to edema takes place most abundantly from the venules. The permeability of the portion of the capillary web that is near the arterioles increases only when the venous pressure rises so

high as to approximate that in the arteries. Under such circumstances the gradient of permeability along the small vessels disappears, the capillaries and venules everywhere leaking fluid. The character of the vital staining developing under such circumstances indicates, like the evidence of previous work, that the cause for the gradient is to be sought in a structural differentiation.

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EXPLANATION OF PLATE 22

FIG. 1. Ears of a mouse after an intravenous injection of pontamine sky blue,—to illustrate some of the changes in permeability when the venous pressure has been raised to a moderate degree. A pressure of 9.4 cm. water was exerted upon the fan vessels of the left ear by means of the apparatus described in the text. The pressure was not great enough to occlude the large veins but compressed them to about half their original diameter and there was an abundant rapid flow past the obstruction. After exerting the pressure for 3 minutes the injection of dye into the circulation was begun, the total quantity being given in half a minute. 3¾ minutes after the end of the injection the pressure was relaxed and the ears were immediately severed and photographed during the next 2 minutes.

It will be seen that in both ears there was a patchy staining. In the control this was slight and it was restricted to the region supplied by the venules and the

furthest portion of the capillary web. In the ear subjected to venous hyperemia the coloration was intense. The dye had escaped in great abundance from the venules and back along the capillaries as well. Some had got out even from the large veins.
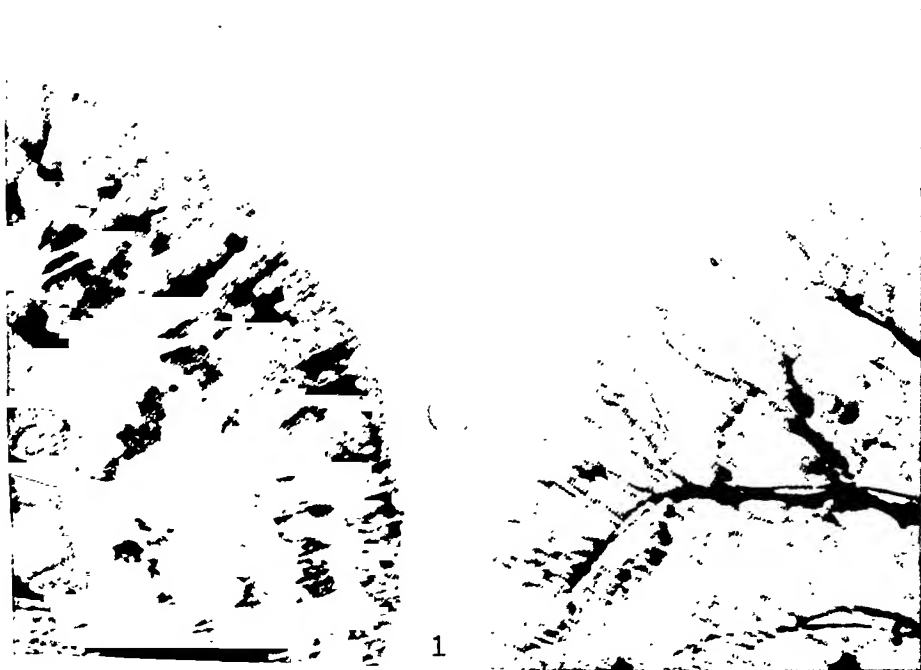
Photographed by Louis Schmidt

(McMaster and Hudack: Vessels involved in hydrostatic transudation)

furthest portion of the capillary web.   In the ear subjected to venous hyperemia the coloration was intense.   The dye had escaped in great abundance from the venules and back along the capillaries as well.   Some had got out even from the large veins.

PLATE 22

Photographed by Louis Schmidt

(McMaster and Hudack: Vessels involved in hydrostatic transudation)

THE GRADIENT OF PERMEABILITY OF THE SKIN VESSELS AS INFLUENCED BY HEAT, COLD, AND LIGHT

By STEPHEN HUDACK, M.D., AND PHILIP D. McMASTER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATE 23

The object of the work here reported has been to determine whether the gradient of vascular permeability demonstrable in the skin of mammals (1) undergoes alteration during active hyperemia, and to learn the consequences to it of vascular injury by heat and cold. The ear of the mouse has been utilized because any alterations of the gradient of permeability during the distribution of vital dyes from the blood is almost diagrammatically visible in an altered color pattern.

General Method

Young mice of 18 to 20 gm. under luminal anesthesia have been employed throughout. After exposure of one of the ears to heat, cold, or light, a dye was injected into a tail vein, and soon thereafter both ears were lopped off with scissors to check the progress of the staining in them. They were at once arranged symmetrically in paraffin oil under the same large cover glass and studied over white porcelain in a combination of transmitted and reflected light. The exposed ear was always cut off first, since it tended to stain the more promptly. In the amputated organ the vascular network was not as sharply outlined as in the living animal, because of the loss of some of the contents of the vessels, but it could still be seen plainly enough for the relations of the staining to arterioles, capillaries, and venules, respectively, to be readily made out.

Pontamine sky blue, a dye that escapes but slowly, was employed as routine because it serves better than more rapidly escaping materials to disclose slight local differences in vascular permeability. For corroboratory tests Chicago blue 6B was injected. A study of photographs taken at once, after the amputation of ears into which pontamine blue had emerged, and 5 minutes later, disclosed no significant extravascular redistribution of the dye. Nevertheless, the comparison of the experimental and control ears was always carried out very rapidly. The technic of the lighting has been described in a previous paper (1).

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Evidence of the Normal Gradient of Vascular Permeability

Ordinarily pontamine blue, Chicago blue, and other vital dyes escape with greatest ease through the walls of the small venules of the corium, and a little less readily through the wall of the adjoining portion of the capillary web, the permeability of the capillaries diminishing greatly in the direction of the arterioles. The influence of these local differences is manifest in the color pattern seen in the ear soon after the dye has been put in circulation. Brilliant patches develop in the region supplied from the venules and the further capillary region when as yet no staining has occurred anywhere else in the ear. The restricted distribution of the dye is not referable to specific affinities for certain sorts of tissue. The tissue indeed is not stained, the dye being contained in the interstitial fluid (2). After a greater or less time, depending on the diffusibility of the dye, the colored patches are lost in a general staining which is partly the result of dye escape from the proximal capillary region, and partly due to a secondary spread through the tissues from the region first stained. Any deviation from the general course of events implies an alteration in the opportunity for materials to pass out of the blood.

The Effects of Heat

The effects of heat on the color pattern were studied in detail.

The anesthetized mouse was placed on its back upon a cork platform beyond which its head extended over two converging test-tubes about 15 cm. long and 2.5 cm. in diameter into which the ears dipped. The edges of the tubes, which were not flared, came within 1 mm. of each other, and one tube had been filled brim full of water at room temperature, while the other contained water that was kept at the desired warmth by means of a small Bunsen flame. The ears were moistened with alcohol to facilitate immersion, and the hair between them on the top of the head was oiled. Under such conditions the tubes remained full after immersion of the ears, even to a bulging of the meniscus, whereas in lack of the oiling, water was drawn out by capillarity and a drip ensued. The head was held free of the tubes, by a clamp on the skin of the lower lip. When the apparatus had been properly arranged both ears were symmetrically immersed nearly to their bases and they did not touch the glass anywhere. The temperature was followed with a thermometer placed immediately next them. After one ear had been heated for a greater or less time, dye was injected into a tail vein of the mouse without altering its position. As routine, 0.1 cc. of isotonic half strength pontamine blue (a 21.6

per cent watery solution of our new preparation of the dye, mixed with an equal part of Locke's solution) was injected in the course of 1 minute. Isotonic half strength Chicago blue 6B (a 17.1 per cent watery solution mixed equally with Locke's solution) was sometimes substituted. The progress of the staining of the ears was followed through a lens, and when the moment for closer inspection seemed to have arrived the animal was lifted away by means of the mosquito forceps and the ears cut off.

Exposure to water at between 42° and 43°C. for 7 minutes caused a pronounced active hyperemia, the ear becoming bright pink; and staining occurred so rapidly as to necessitate amputation of the organ within less than a minute after the end of the dye injection. It was already thickly patched with dark blue on a paler blue ground (Fig. 1.). Very little dye had as yet left the vessels of the control ear. The patches in the heated specimen were far more numerous than ordinarily, as could be seen when the animal was not killed until patching had begun in the control ear; but they were localized to the tissue about the smallest venular trees, just as usual. Seen amidst the general staining they appeared smaller than ordinary. The capillaries were still visible in the amputated specimen because of dye-stained blood within them, as was not the case in the control.

When the heating at 44°C. had been kept up for 14 minutes prior to dye injection, stain escaped abundantly from all of the small vessels, but still in greatest amount from the venules. The tissue had become slightly edematous prior to the injection (Fig. 2). Exposure to 45°C. for 14 minutes caused a more considerable edema. There now developed a deep diffuse staining with but the slightest intensification in the perivenular region and this soon lost (Fig. 3). At 46°C. for 12 minutes the aural muscles contracted, crinkling the ear. Since a circulatory disturbance due to such cause could not be ruled out, the specimens were discarded.

For additional tests the ear was heated by blowing a fine jet of compressed air through the upper part of a Bunsen flame to reach the ear at a distance of 10 to 15 cm. The mouse was laid on its side upon a dais immediately behind a wooden block above which the outer two-thirds of the ear projected; and the head was so placed at the edge of the dais that the control ear and the region about it were not pressed upon. The temperature was taken with a thermometer moved here and there immediately about the ear, throughout the period of exposure.

The ear in the blast of air soon became brightly hyperemic. Heating at 46°C. for 3 minutes, with dye injection during another minute while the exposure was continued, yielded, in yet another minute, a diffuse staining that was deep and even near the edge of the ear, with an intense patching superimposed on somewhat lighter staining further in toward the head, where the tissue was thicker and the vascular disturbance not so great. Patching had just begun at this time in the control ear,—which was not wholly protected from the warm air,—but the patches were relatively few and pale.

Heating at 56°C. for 4 minutes caused an intense local hyperemia with edema and immediate diffuse staining. Toward the base of the ear, where the heating had been less, a brilliant, abundant patching on a background of diffuse color had developed at the time when the ear was lopped off, 3 minutes after the injection of pontamine blue. Lymphatics laden with dye-stained fluid, from the dark blue, edematous tissue further out, coursed through this region. The control ear showed no staining whatever.

A degree of heating which induces active hyperemia without edema brings about several important changes in the staining of the ear. Patching takes place far more quickly than in the control organ; and the patches are more numerous. Also in the case of pontamine blue diffuse coloration develops concurrently with the patching as never happens with this poorly diffusible pigment under normal conditions,—though with highly diffusible dyes it is a regular occurrence (1). Increased blood flow, heightened intracapillary pressure, and the capillary dilatation of active hyperemia are doubtless responsible for the phenomenon.

Heating that suffices to cause slight edema in the hyperemic ear renders the capillary web in the corium everywhere so permeable to pontamine blue and Chicago blue that an intense diffuse staining follows practically at once upon the injection into the blood stream; yet even under such circumstances, with the effective concentration of dye rapidly diminishing through loss to the tissues on the way to the venules, the escape is still greatest from these latter, the color about them being definitely more intense than elsewhere. Only when heat has so damaged the vessels as to cause fulminant edema is the distribution from the blood approximately an even one, as evidenced by the intense general staining.

The Effect of Cold

The cold to which the mouse ear was exposed ranged from that which induced only an active hyperemia to a temperature that caused prompt freezing.

For most of the tests the ear was placed in a current of cold carbon dioxide mixed with air. Care was taken that the organ should be dry, for wet mouse skin lets carbon dioxide through so readily that the tissue pH undergoes alteration in the direction of acidity (3). It was found that when a single large piece of solid carbon dioxide is placed in a funnel held vertically in a ring-stand, and inclosed by inverting upon it another slightly smaller funnel, a continuous jet of cool air emerges from the end of the lower funnel so forcibly as to make itself felt 4 to 5 cm. away. This only happens if the end of the upper funnel is open, for the carbon dioxide evaporates very slowly when no air current can pass through the apparatus. The anesthetized mouse was kept warm while one ear was exposed to the jet. The width of the latter was conditioned, of course, by the funnel opening. It was wide enough for a thermometer to be put next the ear in the cold stream. Complete precision in the degree of cooling was not necessary to the work.

Ears chilled at 1–4°C. for 10 minutes became brightly hyperemic during this period; and on the injection of dye the characteristic patchy staining took place but much more rapidly than in the control, irrespective of whether cooling was continued or not. This happened even when the hyperemia had largely subsided after removal of the ear from the stream. There was also some diffuse staining at a time when none had taken place in the control.

When the ear was placed close to the end of the funnel, where the temperature of the jet was −2°C., it froze within 1 minute. Immediately that this happened it was taken out to thaw; and dye was injected during the subsequent period of intense hyperemia. The ear stained practically at once, with intense perivenular patchings on a ground of diffuse blue, being already deeply colored at a time when the control was practically unstained.

When the outer two-thirds of the ear was frozen at a low temperature and left in this condition for several minutes, the muscle of the affected part contracted on thawing; and though this contraction wore off and the tissue became bright pink, the circulation was imperfect, as appeared when pontamine blue was injected, the dye penetrating with difficulty or not at all into the region that had been frozen. Nearer the base of the ear was a marginal zone which had become markedly edematous and stained rapidly and diffusely, while still nearer the head,

The experiments disclosed incidentally some of the functional changes which precede and are doubtless responsible for the structural alterations characteristic of injury by heat and cold. One such change deserves special mention. The slightest freezing of the mouse ear, at the highest temperature at which this can soon be accomplished (−2°C. in our experience),[1] causes the walls of the large arteries to become readily permeable to dyes (pontamine blue, Chicago blue) which fail to pass through them at all under ordinary circumstances. A zone of deep color soon forms along the outside of the injured vessels. With longer freezing at lower temperatures the arterial leakage is accentuated. Most stuffs carried by the blood are greatly more diffusible than the dyes we used, and the damaged arterial wall must provide but a slight barrier to their escape. The fact is known that after freezing, blood platelets soon collect on the walls of the arteries of the affected part, and that arterial thrombosis is responsible for much of the late damage (11). The media of the arteries undergoes a degeneration. It is plain that a pathological seepage through the vessel wall precedes these changes. Probably it has much to do with them.

SUMMARY

The mounting gradient of permeability along the small vessels of the corium is essentially unaltered by active hyperemia produced by heat, cold, or light. Only when the vascular walls are so damaged that rapid leakage ensues, as shown by the development of edema, does the permeability of the capillary web as a whole approximate that of the venules. It is plain that the normal gradient of vascular permeability depends upon the integrity of the vessel wall.

The method of experiment described can be utilized for a study of the functional changes which result in the lesions due to burning and freezing.

[1] Mammalian tissues freeze at −0 56° to −0.97°C. in the absence of circulation (10). Lewis (4) found that a local temperature of −2.2°C. will freeze the skin of normal human beings.

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EXPLANATION OF PLATE 23

For purposes of comparison the ears have been transposed, so that the marginal regions lie next each other. All have been photographed from above.

FIG. 1. One ear of an 18 gm. female mouse under luminal anesthesia was submerged for 7 minutes in water between 42.0° and 42.6°C. During the next minute 0.1 cc. of half strength pontamine sky blue was injected intravenously. 52 seconds later the heated ear already showed a pronounced staining. Both ears were cut off, placed side by side on a porcelain plaque, and covered with paraffin oil. The picture was taken approximately 3 minutes after the amputation. The heated ear shows a pronounced patchy staining with some general coloration as well. Dye has just begun to escape from the venules of the control.

FIG. 2. Results of heating an ear at 42–44°C. for 14 minutes. Same general technic as in the experiment of Fig. 1. There are still local differences in staining indicative of a gradient of vascular permeability. No dye has escaped as yet into the control ear.

FIG. 3. Results of heating an ear at 45°C. for 14 minutes; technic as in the experiments of Figs. 1 and 2. The fine white dotting is caused by the dispersion of light by the sebaceous glands situated around the hair follicles.
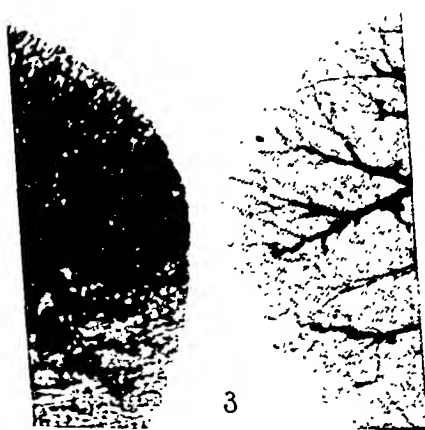
PLATE 23

1

2

3

Photographed by Louis Schmidt

(Hudack and McMaster: Gradient of permeability of skin vessels)

PROPERTIES OF THE CAUSATIVE AGENT OF A CHICKEN TUMOR

II. THE INACTIVATION OF THE TUMOR-PRODUCING AGENT BY MONO-CHROMATIC ULTRA-VIOLET LIGHT*

BY ERNEST STURM, FREDERICK L. GATES, M.D., AND JAMES B. MURPHY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

Early work on Chicken Tumor I indicated that, while the sarcoma cells are as susceptible to ultra-violet radiation as other cells, the etiological agent separable from the cells is relatively resistant (1). Later Baker and Peacock (2) estimated that five times the lethal dose for pathogenic bacteria was not sufficient to destroy the activity of the chicken tumor agent, and that even eight times the amount did not invariably destroy the activity (7). This observation was confirmed in general by Illingworth and Alexander (3). All these observations were made without particular reference to wave length or the absolute energy involved. One of the present authors (Gates (4)), using measured monochromatic light and a standard technique, has been engaged in a comparative study of the energies required to kill or inactivate various organisms or biological agents at single wave lengths in the ultra-violet region. By plotting the energies required against the corresponding wave lengths, similar graphs are obtained for comparing the qualitative and quantitative action of ultra-violet light. This method offers an opportunity to compare the reaction of the tumor agent with that of bacterial cells, virus or phage.[1]

[1] A preliminary report on this work was published in the *Internat. Conf. Cancer* London, 1928, 33.

Method

1 gm. of finely mashed Chicken Tumor I, or an equal amount of tumor desiccate, was emulsified with 10 cc. of water, thoroughly shaken, centrifuged at high speed, and the supernatant fluid passed through a filter paper. The bottom surface of a small Petri plate (4.5 cm. in diameter) was covered with enough melted agar to form a layer 1.5 to 2 mm. thick after solidification.[2] On this smooth surface 2 drops of the thick tumor filtrate was evenly spread and allowed to stand at room temperature for about 45 minutes, when sufficient drying had occurred to prevent flowing of the material. Uniform strips, 3 x 20 mm., were then cut from the middle of the agar plate and exposed to varying doses of ultra-violet radiation. With a quartz mercury arc as the source of energy, the specimens were placed behind the exit slit of a large monochromatic illuminator for various intervals of time. The wave lengths selected lay between λ 238 and λ313 mμ, and the intensity of the radiation at each wave length (measured in ergs per mm.[2] sec. by means of a standardized thermopile and high sensitivity galvanometer) multiplied by the time of exposure gave the total energy per mm.[2] for each exposure. Immediately afterwards the strips were loaded in 16 gauge lumbar puncture needles and injected intradermally in chickens, each chicken receiving also a control unexposed strip which had been kept under the same general conditions as the test specimens.

The uniformity of the control "takes" and the reasonable regularity of results with the exposed specimens indicated that the presence of the neutral agar had no significant effect on the reactions.

RESULTS

The results of 624 irradiation tests are shown graphically in Text-fig. 1. The curve is based on the points at which the agent was attenuated to such an extent that tumors resulted from less than 50 per cent of the test inoculations. The results for λ313 mμ are not shown, as the tumor agent was not inactivated even by an exposure of 80,000 ergs per mm.[2]

Ultra-violet inactivation curves for a bacterium (4), a typical virus (5) and a phage (6) have been plotted in Text-fig. 1 for comparison.

The energy required at each wave length to inactivate the tumor agent is far greater than that required to kill or inactivate bacteria, virus or phage.[3] It is of equal importance to note the relative differ-

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[2] In preliminary experiments carried out to test the suitability of this method, gelatine was used, but as it was found that this substance had a definite enhancing action on the tumor agent, agar was substituted.

[3] Baker and Nanavutty, working with an unresolved ultra-violet spectrum, estimate that phage has the same degree of susceptibility as bacteria, that the chicken

E. STURM, F. L. GATES, AND JAS. B. MURPHY
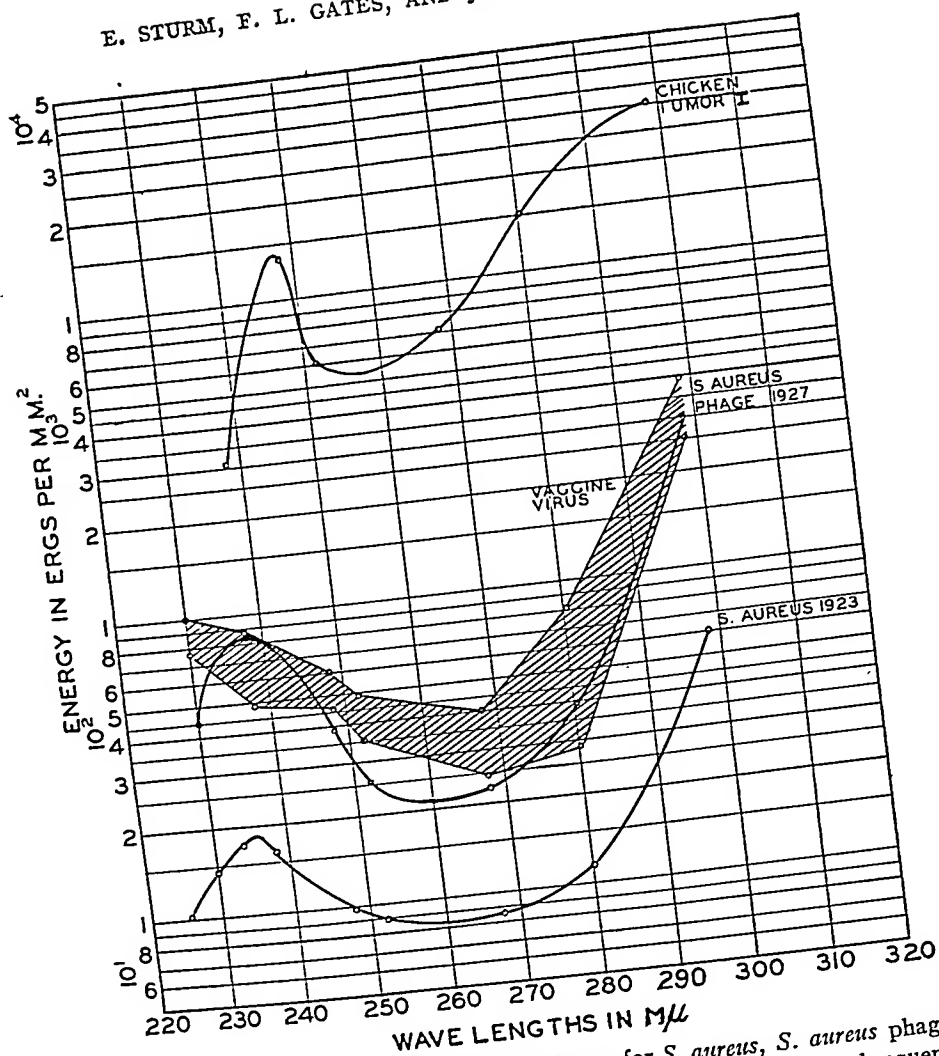
TEXT-FIG. 1. The points on the curves drawn for *S. aureus*, *S. aureus* phage. and Chicken Tumor I represent the energies required to reduce the subsequent colony or plaque formation or tumor takes to 50 per cent of those obtained with control specimens.

The cross-hatched area shows the limits of energy in various experiments which resulted in the failure of exposed vaccine virus to produce lesions in susceptible rabbits.

tumor is 8 times more resistant, while ferments and antibodies are 20 to 120 times more resistant (7).

ences in activity of the various wave lengths examined. The general form of the curves for bacteria, virus and phage is similar. Contrasting these with the curve for the tumor agent, it is seen that among the shorter wave lengths tested the most active for the tumor agent ($\lambda 238$) is least active for the other group; and the least active for the tumor agent ($\lambda\ 248$) is in the range of the most active wave lengths for bacteria, virus and phage.

DISCUSSION AND SUMMARY

Even though part of the energy of the incident light is probably absorbed by chemical entities which play no part in the specific reaction of inactivation, nevertheless the wave lengths most active in destroying biological cells or agents will presumably be found to be among those absorbed in the highest proportion. This would indicate that the curves here presented are approximately reciprocal to the coefficients of absorption of particular substances, the destruction of which caused the inactivation of the agents or the death of the cells. The similarity between the curves for bacteria, virus, and phage, both in shape and in total involved energies, suggests the presence of a common factor, or of closely related chemical entities, sensitive to ultra-violet light, whereas the data for the tumor agent suggest that its inactivation is due to the destruction of a substance having an essentially different spectral absorption, and therefore of a different chemical character. While the amount of ultra-violet energy required to affect the tumor agent is great, it is still less than that involved in the inactivation of some of the enzymes (7).

A study is under way to compare the deduced spectral analysis with the actual coefficients of absorption of the highly purified tumor agent.

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THE ASSOCIATION OF PNEUMOCOCCI, HEMOPHILUS INFLUENZAE, AND STREPTOCOCCUS HEMOLYTICUS WITH CORYZA, PHARYNGITIS, AND SINUSITIS IN MAN

By L. T. WEBSTER, M.D., AND A. D. CLOW

(From the Laboratories of The Rockefeller Institute for Medical Research)

In a previous paper (1) it was reported that persons harboring pneumococci in their noses and throats were relatively subject to coryza, pharyngitis, and sinusitis, and conversely that persons not harboring these organisms were relatively free of these diseases. Moreover, it was stated that the occurrence and degree of symptoms were associated to some extent with variations in the numbers of pneumococci obtained from the nose and throat cultures. These relationships are described in more detail in the present paper.

Technique

The general scheme of study has been described previously (1). The group on which the observations were made comprised adults working at The Rockefeller Institute and in some instances their children. Each individual was questioned every 2 or 3 days to ascertain the presence of fever, chill, malaise, headache, nasal discharge, sore throat, sneezing, cough, etc., and was requested to report whenever such symptoms occurred. Cultures of the nasal passages and throat were usually made weekly; when symptoms were present, however, cultures were taken daily, if possible. Material obtained by means of a sterile swab was streaked over one or two freshly prepared 15 cm. rabbit blood agar plates. The resultant aerobic growth was classified into Gram-positive and negative cocci and bacilli. Pneumococci, S. hemolyticus, and H. influenzae were identified with care (1).

RESULTS

The present analysis is based on the protocols of the individuals studied most intensively. Since considerable information about each case is needed in order to judge the degree of association of upper respiratory tract symptoms and potential pathogens, the selected

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group constitutes less than 20 per cent of the entire number studied. This association, moreover, became increasingly apparent with the amount of care exercised in obtaining full histories, in taking frequent cultures, and examining them minutely.

The protocols are divided into those of individuals not carriers of pneumococci, *H. influenzae*, and *S. hemolyticus*, and individuals who were transient, periodic, and chronic carriers of these organisms. Illustrative cases are described in the following paragraphs.

Non-Carriers.—Two in number. Were free of symptoms $2\frac{1}{2}$ years and 1 year respectively.

Transient Carriers.—

Case 1, free of upper respiratory tract symptoms and pathogens on 25 tests from Sept. 4, 1928, to Nov. 27, 1928, suffered an influenzal attack from Nov. 26, 1928, to Dec. 10, 1928, during the epidemic. At this time, 6 of 32 cultures contained large amounts of *H. influenzae* and 2 contained pneumococci of an unnumbered, specific type. Subsequently, tests were negative and remained so during a 9 day period of sore throat from Feb. 1, 1929, to Feb. 10, 1929. Thereafter no symptoms were reported and 34 cultures contained none of the above mentioned organisms. On Nov. 17, 1930, the individual reported sore throat lasting 24 hours and yielded *H. influenzae* on this date and 1 week later. The succeeding 17 cultures were free of these organisms and no further symptoms were noted until Apr. 20, 1931, at which time chills, weakness, sneezing, nasal discharge, and sore throat were experienced for 2 days and *H. influenzae* was recovered on Apr. 20, 1931. 7 tests thereafter were negative; 1 on Aug. 27, 1931, contained *H. influenzae*; 5 tests then proved negative. On Oct. 19, 1931, chills, fever, malaise, and sore throat developed, lasting 5 days. During this time and thereafter to Nov. 1, 1931, 5 cultures were taken, all negative for the organisms.

Case 55 was a carrier of pneumococci Type XIII on 14 tests from Oct. 11, 1929, to Feb. 7, 1930. During this time one 6 day period of nasal discharge preceded by malaise, nasal obstruction, and sore throat was noted. Tests were negative from Feb. 7 to Apr. 15, 1930. From Apr. 11 to Apr. 22, 1930, the individual suffered a relatively severe attack of prostration, irritability, and general aching sensations, accompanied by sneezing, nasal obstruction, and slight discharge. On Apr. 15 and 16, *S. hemolyticus* was recovered from the throat. Subsequent tests were negative. On Oct. 14, 1931, malaise and nasal congestion were reported, but 5 tests from Sept. 24 to Oct. 27, 1931, were negative. Commencing Oct. 30, 1931, a moderately severe attack of coryza, lasting 8 days, was experienced, and 2 weeks later, a second attack, lasting 10 days, was reported. 7 of 9 cultures taken during this period contained *S. hemolyticus*. Shortly after the symptoms disappeared, the tests became negative and remained so for $2\frac{1}{2}$ months, during which time a 48 hour period of nasal discharge and sore throat

occurred. On Feb. 13, sore throat and nasal discharge were reported, lasting 5 days, and on Feb. 14 and 16 *H. influenzae* was obtained. No further symptoms were reported and subsequent tests were negative for these organisms[1] (Text-fig. 1).

These cases are examples of individuals who, on brief and infrequent occasions, carry a few pneumococci, *H. influenzae*, or *S. hemolyticus*. Their appearance is usually associated with the presence of upper respiratory tract symptoms, although symptoms may occur during a period of negative cultures. The organisms usually decrease in numbers or disappear at varying intervals after the symptoms have subsided.

Periodic Carriers.—

Case 17, free of pneumococci on 34 tests, from January, 1929, to Jan. 17, 1931, and free of upper respiratory symptoms, save for one 6 day attack of coryza and cough which was not studied, developed on Jan. 16, 1931, chill, nasal discharge, sore throat, and cough which persisted 11 days. Cultures taken the day after the onset of symptoms were negative; those taken daily thereafter, to Jan. 30 and Feb. 4 and 9, contained abundant pneumococci of a single, unnamed type, together with *H. influenzae* and *S. hemolyticus*. A second similar attack was reported from Feb. 20, 1931, to Feb. 24, 1931, but only 1 test was made and this proved to be negative. Thereafter, no further symptoms were noted and 21 tests were negative (Text-fig. 2).

Case 50, free of the organisms on 8 cultures over a 5 months' period, developed on Mar. 3, 1931, chills, malaise, headache, and sore throat, lasting 72 hours. Tests Mar. 30, 1931, Apr. 1, Apr. 7, and Apr. 14 revealed pneumococci Types VIII and XXII and *H. influenzae*. 10 subsequent cultures were free of these organisms and no further symptoms have been reported.

Case 51, suffering a severe attack of fever, chills, malaise, nasal obstruction and discharge, sore throat, and cough from Jan. 3, 1931, to Jan. 13, 1931, was first cultured Jan. 8 and 9. Pneumococcus Type V and *H. influenzae* were present. A second attack, with nasal discharge and sore throat, lasted from Feb. 16, 1931, to Mar. 8, 1931, during which time 6 cultures were taken, and was accompanied by the presence of Pneumococcus Type X, *H. influenzae*, and occasional *S. hemolyticus*. During a third 7 day period of coryza, 11 cultures were taken which contained these same organisms. 4 subsequent tests were negative, with the exception of 1 instance, when pneumococci Type X appeared in the culture from

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[1] During the past 17 weeks, Oct. 15, 1931, to time of writing, typical meningococci Type II have been recovered in large numbers and it is not known how long these organisms had been present but unrecognized.

1 - *Hem influenzæ*
S - *S hemolyticus*
L - Left naris
R - Right ·
7pn · Per cent pneumococci en
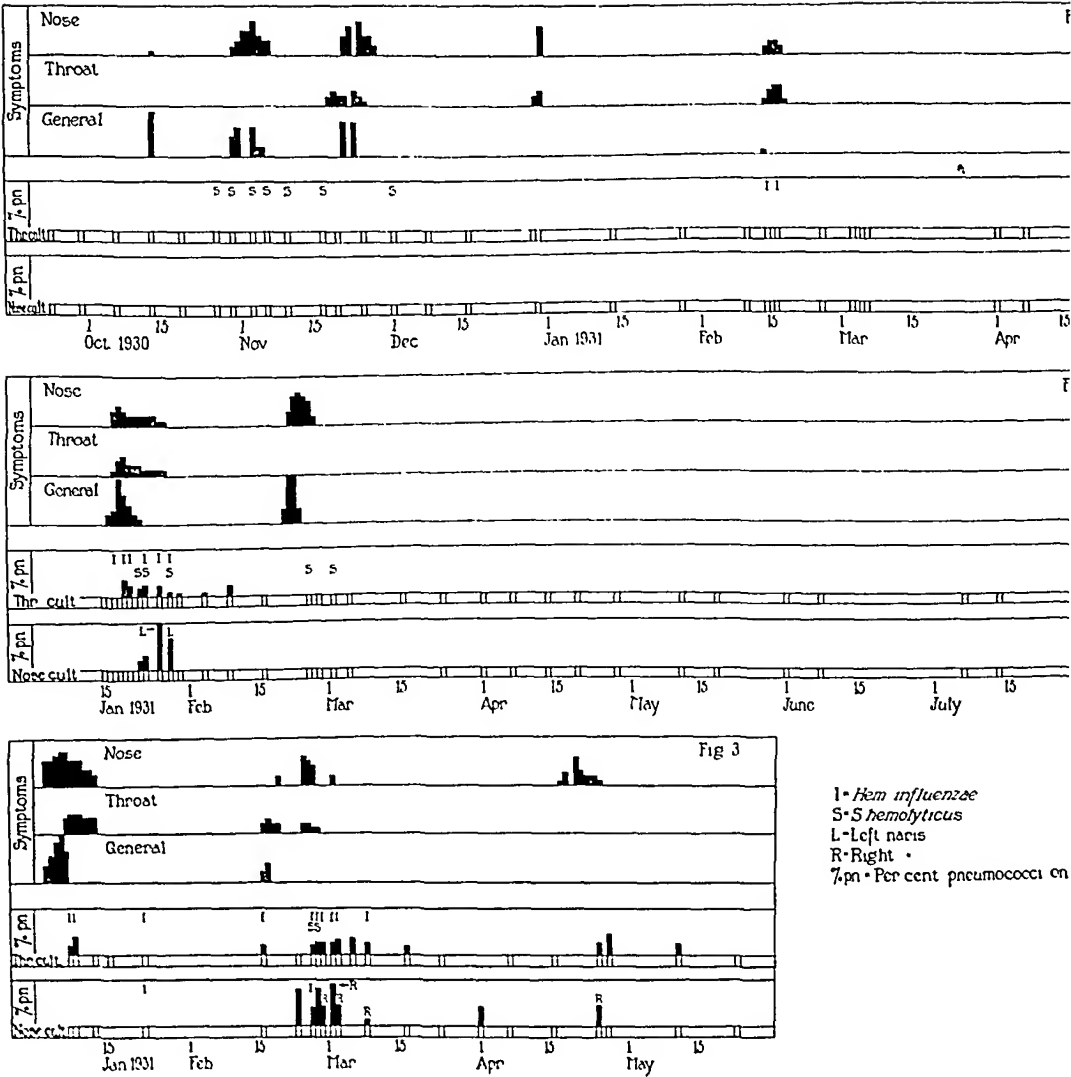
TEXT-FIGS. 1, 2, and 3
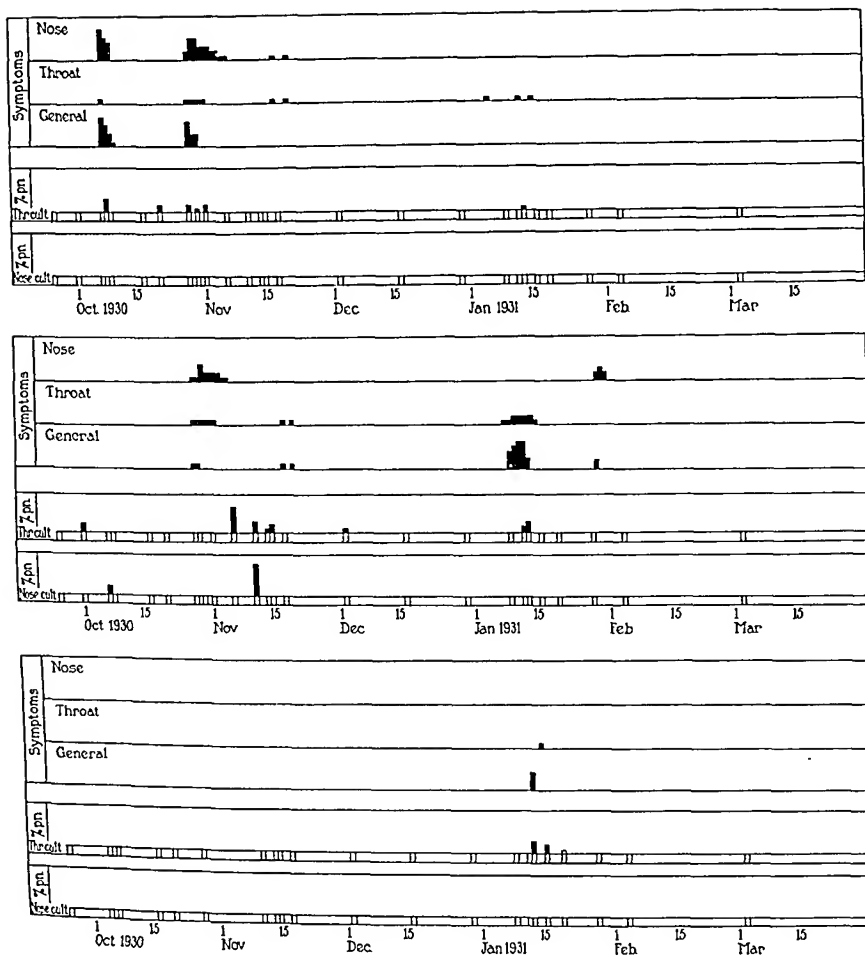
TEXT-FIG. 4

the right naris.   From Apr. 17, 1931, to Apr. 25, nasal congestion and discharge were reported; on Apr. 25 and 27 and May 11, Pneumococcus Type X was recovered.   1 culture taken May 23, 1931, contained none of these organisms (Text-fig. 3).

Cases 33, 34, 35, and 36 are members of a family.   The father, No. 36, has been free of pneumococcus with the exception of 1 brief period, and free of upper respiratory tract symptoms for 3 years.   The mother, No. 35, has been a transient carrier of pneumococci on 3 occasions, during the past 3 years, associated with the appearance of upper respiratory tract symptoms.   The 2 children, Nos. 33 and 34, have carried the same type of pneumococci during 3 winter periods and have experienced 2 or 3 mild attacks of coryza and cough.   The present study was made during the winter of 1930–1931.   One child, No. 33, after 2 negative cultures in September, became a carrier of pneumococci of a specific, unnumbered type on Oct. 10, 1930.   The other child, No. 34, after 3 negative cultures, suffered a mild 3 day attack of coryza, during which pneumococci of the same type appeared on culture.   Subsequently, both children continued to carry this organism on 21 cultures for the remainder of the winter.   They experienced simultaneous attacks of coryza and cough for 2 to 10 days on 3 separate occasions.   During the most severe attack, the mother, No. 35, came down with malaise and sore throat, and yielded pneumococci of the same type as found in the children.   Thereafter, tests became negative and no symptoms were noted until October, 1931 (Text-fig. 4).

In this group of individuals, the organisms appeared during illness and disappeared at varying intervals after the symptoms ceased. *Chronic Carriers.—*

Case 15 has been a carrier of Pneumococcus Type XIII for at least 4 years and Case 52 a carrier of an unnumbered specific type of pneumococcus for at least 1 year.   Each has experienced during the winter months 4 to 6 attacks of frontal headache, malaise, nasal discharge, and sore throat.   On these occasions, roentgenographs have shown extensive clouding of the antra, cultures from the throat show a great increase in numbers of organisms, and cultures from the nares, previously free, show the organisms present often in nearly pure culture.   Moreover, in instances when the individual's pain was localized to one antrum, this localization corresponded to the localization of clouding in the roentgenogram and to the localization of the organisms in right or left nares.   When symptoms subsided, the nasal cultures became free of pneumococci, the throat cultures contained them in fewer numbers, and the roentgenographs showed some clearing of the antra (Text-figs. 5 and 6).

In brief, chronic carriers of pneumococci proved subject to respiratory tract symptoms and antrum disease.   There appeared, moreover, a direct relation between the degree of symptoms, amount of
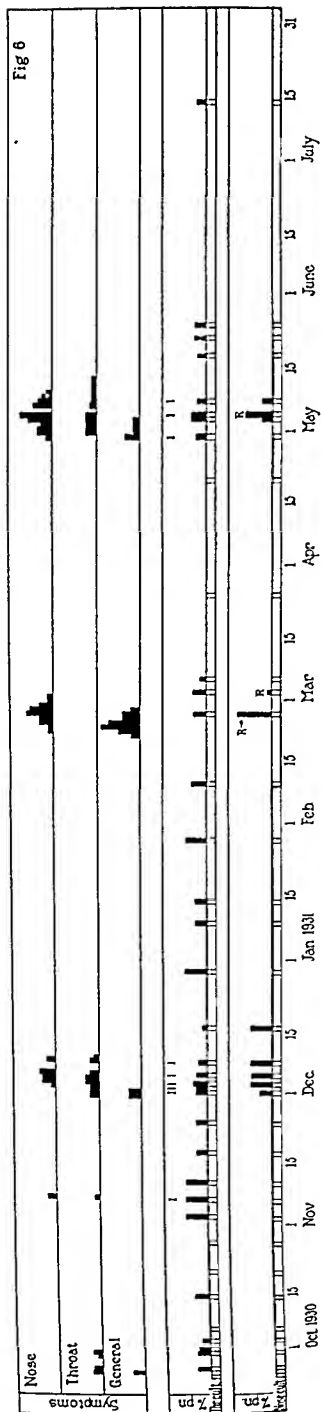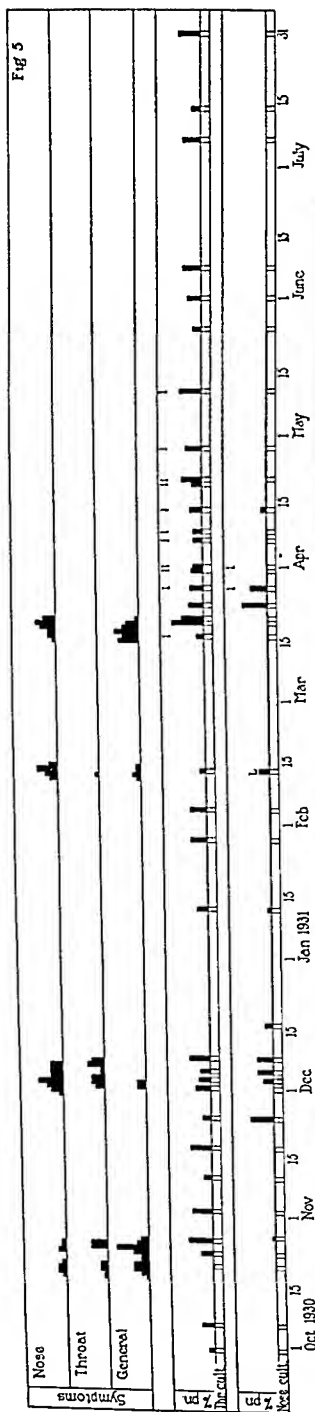
TEXT-FIGS. 5 and 6

clouding of antra in roentgenographs, and numbers of organisms contained on throat and nose cultures.

A relationship between the presence of these pathogens and symptoms, although demonstrated in all cases of this special series, may not invariably exist. Indeed, in three cases studied with less care during 1928 and 1929, no such organisms were found during attacks of upper respiratory tract disease. The limits of this relationship therefore must be decided by painstaking study.

DISCUSSION AND CONCLUSIONS

Pneumococci, *H. influenzae*, and *S. hemolyticus* are known to be frequent inhabitants of the upper respiratory tract, but most workers have not recognized any definite relationships between their presence and coryza, sore throat, influenzal, and sinusitis attacks (2–5). Dochez, Shibley, and Mills, however, in their experimental studies of common cold, state that in both the spontaneous and experimentally induced "colds" in anthropoid apes, the "most significant change observed has been the increase of activity on the part of the potential pathogens habitually present in the throat flora. Coincident with the appearance of symptoms, pneumococci, *S. hemolyticus*, and *B. pfeifferi* have developed in greatly increased numbers and have spread over a wide area of the nasopharyngeal mucous membranes. These organisms became at this time conspicuous even in the nose, where they are seldom or never present under normal conditions. The same phenomena have not been observed in human beings" (6, 7).

The essential facts of the present observations are that persons free of pneumococci, *H. influenzae*, and *S. hemolyticus* are in general free of coryza, sore throat, influenzal and sinus attacks; that persons who are occasional or periodic carriers of these organisms may be negative on tests over long healthy periods, but generally become positive during or following attacks and subsequently become negative again; finally, that persons who are chronic carriers show during these illnesses increasing numbers of organisms in the throat and extension of the organisms to the nose.

That these organisms may be the actual incitants has been claimed by Park (8); that they are secondary invaders is the view of Shibley,

Mills, and Dochez who state as a result of their experimental work on this subject that "the most important significance of viruses of this type [common cold] seems to lie in their capacity to incite activity on the part of the more dangerous pathogenic organisms that infect the upper respiratory tract" (7). The present observations bring out the intimate relationship between these pathogens and upper respiratory tract symptoms, but do not disclose the nature of this relationship.

Finally, an addition has been made to the knowledge of the mode of spread of these organisms. A focus of growth and dissemination has been determined in the nasal passages and throat of individuals with chronic upper respiratory tract disease and increases in numbers of the organisms at the focus and their spread to contacts have been related to the winter season and to the occurrence of symptoms in the carrier. The observations suggest that the dosage of these organisms in a community is controlled by the resistance of the carrier and of the contacts. This view is in agreement with the facts derived from studies of native animal infections (9).

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THE EFFECT OF TESTICLE EXTRACT ON THE GROWTH OF TRANSPLANTABLE MOUSE TUMORS*

By RADFORD C. TANZER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

The recent work of Duran-Reynals (1) has demonstrated that simple water extracts of rat and rabbit testicle, when brought in contact with cell suspensions of the Brown-Pearce rabbit tumor, exert a definite inhibiting effect upon the transplantability and growth of cells from such suspensions in the intradermal tissues of rabbits. This action, which can also be produced by an almost protein-free fraction, is in sharp contrast to the recognized power of extracts of testicle and, to a minor degree, of skin and some other organs, to enhance many bacterial and virus infections (2–7). It has seemed desirable to test the action of these organ extracts upon other groups of tumors and to determine more definitely the nature of such activity.

The Effect of Testicle Extract on Spontaneous Tumors

The first material utilized consisted of tumors occurring spontaneously in the strains of mice propagated in this laboratory.

The tumors varied considerably in size and location, but microscopically all fell into the class of simple or papillomatous adenocarcinomata. They were excised under aseptic conditions with as little damage as possible to the host and, after the healthy tumor material was separated from the surrounding tissue and freed from necrotic areas, it was treated in one of two ways. In the first fifteen instances the material was minced, passed through a double layer of sterile gauze, and then halved. One portion was mixed with an equal volume of a Ringer's solution extract of normal rat testicle, and the other with the same amount of plain Ringer's solution as control. Testicle extract was prepared in the following manner: Adult rats were chloroformed, bled, and the testes removed aseptically. The pulp was ground with sand, extracted with Ringer's solution, 1 cc. per gram

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* This investigation was carried on by means of funds from the Rutherford Donation.

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of pulp, and centrifuged. The supernatant fluid, or so called testicle extract, was used within 3 hours.

Both suspensions were incubated at 37°C. for 2 or 2½ hours and reinjected intradermally in corresponding regions of the abdomen or chest wall of the mouse from which the tumor had been removed. The growth of the tumors was measured weekly. The animals died or were killed from 13 to 62 days after injection. The nature of the tumor was confirmed histologically in every case.

In the second group of 20 mice, the procedure was the same as that described above, except that grafts of the tumor tissue about 3 mm. in diameter were used instead of cell suspensions. These were immersed in 1 cc. of testicle extract and,

TABLE I

Effect of Testicle Extract on Autografts of Spontaneous Tumors
A. Influence on Growth

Testicle extract plus	Growth of test tumor. No growth of control	Larger growth than control	Same growth as control	Smaller growth than control	No growth of test tumor. Growth of control
Tumor cell suspension...............	1	1	0	4	2
Tumor graft........................	0	5	4	5	1

B. Influence on Transplantability

Type of transplant	No. mice inoculated	No. successful inoculations	Successful inoculations
			per cent
Tumor suspension + Ringer's..............	15	7	47
Tumor suspension + T. E................	15	6	40
Tumor graft + Ringer's.................	20	15	75
Tumor graft + T. E.....................	20	14	70

after 1 hour incubation, were inoculated into the original host. The grafts for control were incubated in Ringer's solution for the same length of time before inoculation.

The results of these two experiments, in which twenty-three of the thirty-five inoculations resulted in tumors, are shown in Table I.

While the treated tumor material gave a smaller number of takes and the tumors grew somewhat more slowly than the controls in some instances, the difference was not of sufficient magnitude to be considered of importance. Histological examination of all tumors failed

to show any difference between the tumors arising from the treated grafts and the controls.

The Effect of Testicle Extract on Transplanted Tumors

Bashford Carcinoma 63.—The next tests were carried out with a transplantable adenocarcinoma, the Bashford 63.

This tumor of relatively low malignancy was giving at the time of the experiments about 75 per cent of takes on routine transplantation. Preliminary tests showed that the tumor was not easily transplanted by cell suspensions. Therefore, in the twelve experiments, grafts of the tumor about 3 mm. in diameter were

TABLE II

Effect of Rat Testicle Extract upon Bashford Carcinoma

A. Influence on Growth Rate

Type of transplant	No. experiment	No. mice successfully inoculated	Growth of test tumor. No growth of control	Larger growth than control	Same growth as control	Smaller growth than control	No growth of test tumor. Growth of control
Tumor graft plus testicle extract....	12	59	9	6	16	18	10

B. Influence on Transplantability

Type of tumor	No. mice inoculated	Tumor + Ringer's solution			Tumor + testicle extract		
		No. successful inoculations	Successful inoculations per cent	Average diameter cm.	No. successful inoculations	Successful inoculations per cent	Average diameter cm.
Graft....................	74	43	58	1.28	42	57	1.08
Bolting cloth emulsion............	20	1	5		1	5	

used. Before immersion in testicle extract or Ringer's solution these were cut through in several places so as to give a greater surface of exposure. The test grafts were kept in the extract and the controls in Ringer's solution for 30 to 90 minutes at 37°C., except in two experiments in which they remained at room temperature. The results of inoculation of these materials into 94 mice are shown in Table II.

The percentage of successful transplants from test and control grafts was almost identical, indicating the absence of any demonstrable effect of the testicle extract.

Mouse Sarcoma S/37.—A transplantable mouse sarcoma, known as S/37, was used in the next group of experiments.

This tumor regularly gives 100 per cent takes, develops rapidly, and usually ulcerates between the 12th and 16th day. A *brei* was prepared from healthy tumor tissue by passing it through a masher with 1 mm. apertures. Half of the material was placed in testicle extract and the other half in Ringer's solution. The time of contact varied in the different experiments between 30 minutes and 2½ hours. In two of the six experiments the mixtures were kept at 37°C. while the others remained at room temperature. Each of the 48 mice was inoculated with both the tumor tissue after contact with testicle extract and that which had been kept in Ringer's solution.

All of the inoculations resulted in tumors and there was no significant difference in size between the test and the control ones.

Attenuated S/37.—It seemed possible that the unusual malignancy of the S/37 tumor might mask a potential inhibiting factor in testicle extract. To test this point advantage was taken of the observation that the growth rate of this tumor is reduced if the grafts are immersed before inoculation in a fluid the pH of which is varied in either direction from the neutral point. In fact the growth rate of grafts treated in this way is inversely proportional to the degree of deviation of the hydrogen ion concentration of the fluid (unpublished observation).

Healthy S/37 tumor tissue was first passed through a masher, then suspended in a few drops of Ringer's solution and squeezed through sterile bolting cloth. By this process the tumor was broken up into small clumps comprised of not more than thirty or forty cells each. The suspensions were divided into two equal portions, one of which was diluted with an equal volume of testicle extract and the other with an equal volume of Ringer's solution. In some of the experiments a Berkefeld filtrate of testicle extract was used. The fluids of the two cell suspensions, testicle extract and Ringer's solution, were adjusted to a pH which had previously been shown to attenuate, without completely suppressing, the growth of the grafts. In part of the experiments the pH was adjusted to 5.2 by the addition of an acetate buffer, and in the others to 8.4 with a phosphate or boric acid buffer. The tumor cells were kept in this adjusted mixture for 75 minutes at room temperature.

The above described procedure was varied in two additional groups of experiments. In the first, the Ringer's solution in which the tumor cells were suspended was adjusted to a pH of 5.2. After about an hour this suspension was divided into two portions, one diluted with an equal amount of testicle extract and the other with Ringer's solution, and then immediately injected into mice. In the second group the growth activity of the tumors was reduced by heating at 45°C. for 5 to

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15 minutes, after which the tumor cells were suspended in testicle extract or Ringer's solution for a period before inoculation.

The results of all the experiments with modified S/37 are shown in Table III.

It is evident from the figures in Table III that contact with either a plain testicle extract or its filtrate adversely affected the transplanta-

TABLE III

Effect of Testicle Extract on Modified Mouse Sarcoma S/37

Group	Modification of tumor	Type of testicle extract	No. experiments	No. mice inoculated	Growth of test tumor. No growth of control	Larger growth than control	Same growth as control	Smaller growth than control	No growth of test tumor. Growth of control
I	pH 8.4	Plain	2	11	0	1	3	7	0
II	pH 5.2	"	6	30	0	3	9	5	13
III	pH 8.2	Berkefeld filtrate	1	6	0	0	0	0	6
IV	pH 5.2	" "	4	23	0	4	2	9	8
V*	pH 5.2	" "	3	14	0	0	4	7	3
Total I–V......			16	84	0	8	18	28	30
VI	45°C. for 5–15 min.	Plain	4	30	2	0	5	11	12
Total I–VI.....			20	114	2	8	23	39	42

*In this group the tumor was divided into test and control portions after modification, and contact with testicle extract was reduced to a minimum.

bility and growth rate of the modified tumor. Inasmuch as the mixtures of tumor and testicle extract in some cases required slightly more buffer than the control, one might assume this to be a possible cause of the results. Such is not the case, however, as experiments in Group V show. Here modification of the pH was accomplished before the suspension was divided into test and control portions, thus eliminating variations due to the buffer; yet the inhibiting effect remained. This group furnished an even more stringent test than the others, in that

the period of contact with testicle extract outside of the body covered only the time necessary for inoculation. The results suggest that a very short exposure suffices to produce an inhibitory effect. The action of testicle extract on the tumor attenuated by heat again gave evidence of the inhibitory action.

Mouse Sarcoma 180.—It was suggested above that the failure of the testicle extract to influence the growth of the unmodified S/37 was due to its extreme malignancy. That this was probably the correct deduction was indicated by the results with the attenuated tumor. To test this point further the same experimental procedure has been carried out with a mouse sarcoma of a lower grade of malignancy. This tumor, No. 180, grows well, takes in well over 90 per cent of cases, and is

TABLE IV

Effect of Testicle Extract on Mouse Sarcoma 180

Tumor emulsion plus	No. experiments	No. mice inoculated	Growth of test tumor. No growth of control	Larger growth than control	Same growth as control	Smaller growth than control	No growth of test tumor. Growth of control
Plain testicle extract...............	6	29	2	2	5	15	5
Berkefeld filtrate..................	2	12		2	3	4	3
Total......................	8	41	2	4	8	19	8

principally notable for the fact that the usual methods of induction of immunity fail to influence its growth.

The same technique was used in the preparation of the tumor material and the testicle extract as that described above, except that there was no modification of the pH of the solutions. The suspensions were kept at room temperature for 75 minutes. Each mouse received an injection of both the testicle extract suspension and the control suspension in Ringer's solution.

The results, which show a definite inhibiting action of the testicle extract on Sarcoma 180, are shown in Table IV.

Effect of Purified Testicle Extract on Mouse Tumors

The inhibiting action of rat testicle extract on Mouse Sarcoma 180 and the attenuated S/37 is definitely shown by the findings here

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reported. Duran-Reynals has reported that a purified fraction of testicle extract inhibits the growth of the Brown-Pearce rabbit tumor in much the same degree as the full extract (1). This fraction[1] is known to contain in concentrated form the so called Reynals factor which increases the intradermal spread of injected materials and enhances the action of infectious agents. The effect of this fraction was next tested on mouse tumors.

TABLE V

Effect of Purified Testicle Extract on Sarcomata S/37 and 180

Type tumor	No. experiments	No. mice inoculated	Larger growth than control	Same growth as control	Smaller growth than control	No growth of test tumor. Growth of control
S/37	1	7	2	7	2	1
S/37 pH 5.2	2	14		10	6	
180	4	21	6	8	8	1
Total........	7	42	8	25		

This group of experiments included tests on S/37 and its modified strain as well as on Sarcoma 180. The technique was the same as that described in the foregoing experiments, except that the purified fraction was used instead of the full testicle extract. The results as shown in Table V yield no evidence of augmentation or inhibition of tumor growth.

Effect of Heat on the Inhibiting Property of Testicle Extract

The "spreading factor" in plain testicle extract is completely inactivated at a temperature of 60°C. (1, 3), though purified extracts retain their activity even after boiling. If increase in tumor cell permeability were the factor causing inhibition of growth, one would expect this property to be lost on heating.

Testicle extract was heated at 60°, 80°, and 100° for 10 minutes, then mixed with tumor emulsions as in previous experiments. As there was no diminution in the inhibiting action of the extracts heated

[1] The details of the purification technique will be published later. The fraction in 0.4 gm. has the enhancement value of 100 gm. of testicle material.

at different temperatures, the results are tabulated together in Table VI. A control group in which muscle extract was used instead of testicle extract showed no inhibiting action. In fact the tumors treated with this material grew somewhat more rapidly than the controls.

TABLE VI

Effect of Heated Testicle Extract on Sarcomata S/37 and 180

Type of tumor	Type of extract	No. inoculated	Same growth as control	Smaller growth than control	No growth of test tumor. Growth of control
180	Heated 60–100°C.	14	3	8	3
Modified S/37	" 60–100° "	12	7	3	2
Total..........		26	10	11	5

DISCUSSION

The experiments reported above show that testicle extract will inhibit the growth of a mouse sarcoma of a moderate degree of malignancy and an attenuated strain of another more highly malignant sarcoma. The manner in which the testicle extract exerts its depressant effect on the tumors is not clear. Devitalization of the tumor cells, or the stimulation of resistance in the host, or a combined effect are the possibilities to be considered. There is much evidence that the Reynals factor in testicle extract, which enhances the action of infectious agents, produces its effect at least in part by increasing tissue and cell permeability (4, 8, 9). The fact that the fraction of testicle extract containing this factor in purified form was just as effective as the whole extract in inhibiting the Brown-Pearce rabbit tumor led Reynals to suggest that the tumor inhibiting and infection enhancing influences are manifestations of the action of a single agent. However, in the present experiments there is no evidence that the purified fraction has any effect on the mouse tumors. The question raised by this finding must receive further investigations before a conclusion can be reached.

The fact that the tumor-inhibiting property of testicle extract is not destroyed by heat, while the infection-enhancing power is destroyed, adds another point of difference. However, it has been shown that the Reynals factor freed from much of the inert testicle protein is itself

heat-stable. It may well be that the heat inactivation of the crude extract is due to a temporary adsorption of the factor on the coagulated protein, and that in the experiments with tumors there is a gradual release after the material is inoculated into the body.

The relationship of the Reynals factor to the tumor-inhibiting property of testicle extract is not definitely determined by the present work, nor have we sufficient evidence to indicate that the phenomenon here described is due to the same mechanism as the inhibition of Chicken Tumor I (10).

SUMMARY

Grafts of a transplantable mouse sarcoma designated as No. 180, and those of an attenuated strain of a more malignant Sarcoma S/37, treated with testicle extract, either fail to grow on inoculation or result in tumors of a lower growth rate than the controls. Autografts of spontaneous mouse tumors so treated show little if any effect, while the Bashford adenocarcinoma and the unattenuated S/37 are unaffected. The factor in testicle extract responsible for the retarding activity passes readily through a Berkefeld filter and is thermostable.

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STUDIES ON THE NATURE OF THE AGENT TRANSMITTING LEUCOSIS OF FOWLS*

I. Its Concentration in Blood Cells and Plasma and Relation to the Incubation Period

By J. FURTH, M.D.

With the Assistance of Charles Breedis and Ruth Klingelhofer

(*From The Henry Phipps Institute, University of Pennsylvania, Philadelphia*)

INTRODUCTION

The discoveries of Ellermann and Bang (1) and of Rous (2) opened a new field of investigation by disclosing the existence of ultramicroscopic agents capable of producing a variety of neoplastic diseases in fowls. The cytological character of these conditions is specific for each transmissible agent. The nature of these agents is subject to much debate, which has been based mainly on studies of avian tumors initiated by Rous and his associates (3). The studies on leucosis of fowls reported by Ellermann and Bang have been confirmed only in part and little is known concerning the nature of the virus of Ellermann.

Ellermann and Bang observed the development of lymphoid and myeloid leucemia, lymphoid and myeloid tumors, and anemias of varying intensity among chickens inoculated with leucemic material. They did not, however, control the possibility that some of these conditions occurred spontaneously among their laboratory animals. They believed that the type of leucosis resulting from the inoculation was independent of that from which the injected material was obtained. They observed that the agent transmitting leucosis occasionally passed Berkefeld filters and concluded therefore that it is a filterable virus. A recent investigation (4, 5) failed to yield sufficient evidence supporting the view of Ellermann and Bang that lymphoid leucosis, an apparently common condition in the fowl, is caused by the same agent that transmits myeloid leucosis and erythro-

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leucosis.[1] The filterability of the agent of Ellermann and Bang was confirmed (4, 6).

The investigations to be reported in this and in three succeeding papers reveal a close similarity between the behavior of the agent causing leucosis of fowls and those causing avian tumors. Both share some of the properties of viruses that cause infectious diseases. Apparently we are dealing with a single group of agents capable of producing neoplastic conditions; the exact position of these agents in the organic world is the subject of future research.

Material of Study, Technique.—The origin of the transmissible strain investigated, its transfer and methods of blood examination have been described in a previous report (4b). All birds inoculated were Barred Plymouth Rock chickens about 4 or 5 months old. Blood smears were taken frequently from all fowls that appeared sick, and from the entire flock at intervals of from 2 to 4 weeks. Fowls showing no leucemic change within 4 to 5 months after inoculation were killed. All fowls were autopsied but the organs were examined microscopically only when blood smear and gross postmortem appearance did not reveal the cause of death.

Interpretation of Observations.—Observations already described (4) have shown that erythroleucosis and myeloid leucemia are caused by the transmitting agent but its relation to other similar conditions such as lymphoid leucosis was not demonstrable. The three cases of lymphoid leucosis observed in the present study will be grouped among the unsuccessful inoculations but their occurrence will be mentioned in the corresponding series. Myeloma was not observed in these series. Myeloid hyperplasia, associated not with leucemia but with infectious processes (cf. 7), will be included among the unsuccessful inoculations. Mild anemia, without the microscopic changes characteristic of erythroleucosis, if caused apparently by an accompanying infection or injury, will be recorded as "negative." Mild transient anemia also occurred in several fowls during the expected period of incubation. Although these anemias may be analogous to tumor "takes" with regression, or to unapparent or concealed infections, there are no available criteria to differentiate them from ordinary anemias; they will be listed among the unsuccessful inoculations. In several instances the blood smear for a varying period of time was characteristic for erythroleucosis (cf. 8) but finally returned to normal. These cases are considered as erythroleucosis ending in recovery and they are listed among the successful inoculations. The occurrence of severe chronic anemia will be mentioned (cf. 9).

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[1] Erythroleucosis is a severe anemia characterized by numerous lymphoid erythroblasts in the larger vessels and by a selective accumulation of such cells in the bone marrow, liver, and spleen.

The Concentration of the Transmitting Agent in Blood Cells and in Plasma

There are many pitfalls in the study of viruses if quantitative relations are ignored; for example, failure or success in demonstrating filterability may depend chiefly on the initial concentration of the virus. Hitherto quantitative tests of the agent transmitting leucosis have not been feasible, but they were made possible by the increase in virulence of the transmissible strain under investigation. The studies are limited by the following circumstances: (a) Live fowl is the only reagent known for detecting the transmitting agent; (b) each must be observed from 1 to 5 months; (c) the concentration of the transmitting agent in the blood varied within wide limits, the minimal amount producing leucosis ranging between 1 and

TABLE I

Inoculations with Diminishing Amounts of Leucemic Plasma and Blood Cells Passage VII C on August 1, 1930

	Plasma			Blood cells		
	0.5 cc.	0.01 cc.	0.0002 cc.	0.5 cc.	0.01 cc.	0.0002 cc.
No. of fowls inoculated................	4	4	4	4	4	4
No. of successful inoculations...........	3	0	0	1	2	0
No. of fowls that died of intercurrent disease.	0	0	0	1	0	0
Fowls successfully inoculated:						
Average period of incubation, *days*......	88	—	—	75	64	—
Average duration of illness, *days*........	22	—	—	13	26	—

10^{-6} cc.; (d) when injected with some constituent of the blood or with blood sub- jected to certain procedures, only a few of the inoculated fowls develop leucosis.

A series of experiments were performed to determine the concentra- tion of the transmitting agent in the whole blood, in the plasma, and in suspensions of blood cells. These titrations extended over a period of about 8 months and were made in part for the purpose of determining the initial concentration of the transmitting agent in particular samples before subjecting them to various procedures. Two titrations will be recorded in Tables I and II. Table I gives the result of a titration during Passage VII of the transmissible strain studied, Table II that of Passage XIV made about 6 months later. The essential data of these two experiments will then be summarized in Table III, in which are recorded titrations of sera from nineteen leucemic fowls.

The blood count of the donor (No. 815) before transfer was as follows: white blood cells 1,335,000, red blood cells 1,035,000 and hemoglobin 23 (Sahli).

The whole blood, to which 15 per cent heparin solution (1:1000) was added to prevent clotting, was centrifugalized at 2000 R.P.M. for 5 minutes and the clear plasma was recentrifugalized twice in succession at 1800 R.P.M. Contamination of the plasma with cells was avoided while transferring it into clean centrifuge tubes. The blood cells were suspended in a volume of Locke solution corresponding to the amount of plasma removed.

The amount of cells is given in terms of the original volume of whole blood. The smallest volume of cell suspension injected (0.0002 cc.) is estimated to have contained about 260,000 leucocytes, among which over 200 cells were in mitotic division.

One of the four fowls injected with 0.0002 cc. of cell suspension had mild transient anemia, and another multiple lymphoma.

TABLE II

Inoculations with Diminishing Amounts of Leucemic Plasma and Blood Cells Passage XIV B on January 20, 1931

	Plasma			Blood cells		
	10^{-2} cc.	10^{-5} cc.	10^{-7} cc.	10^{-2} cc.	10^{-5} cc.	10^{-7} cc.
No. of fowls inoculated....................	4	4	4	3	4	4
No. of successful inoculations.............	1	1	0	2	2	0
Fowls successfully inoculated:						
Average period of incubation, *days*.......	39	56	—	34	38	—
Average duration of illness, *days*..........	13	94	—	22	39	—

The plasma contained about 20 per cent heparin solution (1:1000). When placed in the counting chamber, the plasma appeared cell-free. The amount of cells is given in terms of packed cells. When counted in the blood-counting chamber, 10^{-5} cc. of cell suspension contained 50,000 leucocytes and a slightly larger number of erythrocytes.

[/] Tumor of the ovary (probably endothelioma) was an incidental finding at the autopsy of one of four fowls injected with 10^{-7} cc. of plasma. One of the four fowls (No. 1526) inoculated with 10^{-7} cc. of cell suspension showed the blood picture of anemia and transient lymphoid leucemia (white blood cells 159,000). It died with widespread, large, round cell sarcomatosis 113 days after inoculation. The illness was observed during 42 days. Failure to produce leucosis in any of the ten fowls injected with blood and suspension of tumor cells of this fowl supports the view that this condition, the first case of leucosarcoma (Sternberg) observed in the fowl, was probably not caused by the transmitting agent.

When Table I and Table II are compared, it becomes evident that in Passage XIV, smaller amounts of both plasma and cells were sufficient to transmit leucosis than in Passage VII. This difference may be due either to an increase in virulence or to an increase in concentration of the transmitting agent. Unfortunately titration of plasma was omitted in the course of the first seven passages, but in the subsequent passages tested transmitting agent was present in the plasma in a very high concentration.

All these transfers were made from pronounced cases of leucosis. Fowls dying of intercurrent diseases are not given among the number inoculated. The amount of cells is given in terms of original whole blood. The plasma was obtained in most instances by three successive centrifugalizations at about 1000 to 2000 R.P.M. for 5 to 15 minutes. When examined in the counting chamber the plasma was usually free from cells even after the second spinning.

From Table III it is evident that leucemic plasma behaves differently from suspensions of cells.

When injected in larger amounts (0.1 cc. or more) the plasma causes leucosis in as many birds as whole blood or a suspension of blood cells, namely in about 55 per cent of all the fowls inoculated. Smaller amounts of plasma cause leucosis only in from 20 to 28.6 per cent of the fowls inoculated, the number of successful inoculations being within a very wide range (10^{-1} to 10^{-6} cc.) apparently independent of the amount injected. The concentration of the transmitting agent in a suspension of cells, as determined by titration, seems to be about as high as in the plasma, the success of the inoculations disappearing with blood cells at 0.000001 cc. and with plasma at 0.0000001 cc. However, amounts of cells smaller than about 0.1 cc. (in terms of original whole blood) produce leucosis in higher percentages of inoculated fowls (33.3 to 71.4 per cent) than the corresponding amounts of plasma.

When the results of single titrations are studied, a difference in the behavior of plasma and cell suspension will again be noted. Success of inoculation with plasma seems to bear a definite relation to the amount of material inoculated. In inoculations with cells this relation is apparently obscured by other factors; e.g., in Passage XII C 0.001 cc. of the suspension of cells caused leucosis in both fowls injected, whereas the inoculations of 0.1 cc. of the same cell suspension in three fowls and 0.00001 cc. into three fowls was without effect.

The Concentration of the Transmitting Agent in Whole Blood, Plasma, and Cell Suspensions

No. of passage	Date of inoculations	Material injected	Amounts inoculated														
			2 to 15 cc.		0.1 to 1.5 cc.		0.01 to 0.09 cc.		0.001 to 0.009 cc.		0.0001 to 0.0009 cc.		0.00001 to 0.00009 cc.		0.000001 cc.		0.0000001 cc.
			No. inoculated	No. of successful inoculations	No. inoculated	No. of successful inoculations	No. inoculated	No. of successful inoculations	No. inoculated	No. of successful inoculations	No. inoculated	No. of successful inoculations	No. inoculated	No. of successful inoculations	No. inoculated	No. of successful inoculations	No. inoculated	No. of successful inoculations
VI C	Apr. 19, 1930	Blood cells			2*	0	3	1										
VII B	June 25, 1930	Whole blood			5	4	3	0										
VII C**	Aug. 1, 1930	Blood cells			4	1	4	2	4	0								
		Plasma			4	3	4	0	4	0								
VII D	Aug. 14, 1930	Whole blood			4	2												
		Blood cells			4	2			4	1								
		Plasma			4	2			3	1								
VII G	Oct. 18, 1930	Whole blood			3	3			2	2								
VIII C	July 25, 1930	Whole blood			6	1	5	3			6	0						
VIII E	Sept. 13, 1930	Whole blood			3	3			2	1			2	1				
		Blood cells			2	1			1	1								
		Plasma			3	1			4	1								
IX B	Oct. 1, 1930	Blood cells			4	3					4	3	2	0				
		Plasma			4	4					4	1						

	Date	Type																		
X B	Sept. 20, 1930	Whole blood		4	2		4		4 4	2 2					4 4	0 1			
		Blood cells		3	2		4		4	2									
		Plasma		7	2														
X C	Sept. 23, 1930	Blood cells		3	2		4	4		1				2					
		Plasma		2	2														
X D	Oct. 18, 1930	Whole blood		4 3	2		2		2										
XI F	Jan. 19, 1931	Whole blood	4	2	0						3	1							
XII A	Oct. 24, 1930	Whole blood	2	4			2				1	0							
XII B	Oct. 29, 1930	Blood cells		3	2	3	3												
		Plasma		2	1	2	1												
XII C	Nov. 8, 1930	Blood cells		3	0			2	2			3	0					
		Plasma		2	1														
XII D	Nov. 22, 1930	Blood cells		2	1			3	2			3	2					
		Plasma		2	1								3	1				
XIII D	Dec. 16, 1930	Whole blood	1	3	2	4		2	2									
XIII F**	Feb. 16, 1931	Blood cells		6	5			6	2									
XIV B	Jan. 8, 1931	Blood cells		3	2	4	1	1		4	2	4	1		4	0	4	0
		Plasma																

* In Passage VI C one of the fowls inoculated with 0.2 cc. cell suspension (No. 816) died of lymphoid leucosis. An attempted transfer of this condition to eight fowls was unsuccessful.

** More data on these transfers may be found in Table I (Passage VII C), Table II (Passage XIV B).

TABLE III—Concluded

			Amounts inoculated														
No. of passage	Date of inoculations	Material injected	2 to 15 cc.		0.1 to 1.5 cc.		0.01 to 0.09 cc.		0.001 to 0.009 cc.		0.0001 to 0.0009 cc.		0.00001 to 0.00009 cc.		0.000001 cc.		0.0000001 cc.	
			No. inoculated	No. of successful inoculations	No. inoculated	No. of successful inoculations	No. inoculated	No. of successful inoculations	No. inoculated	No. of successful inoculations	No. inoculated	No. of successful inoculations	No. inoculated	No. of successful inoculations	No. inoculated	No. of successful inoculations	No. inoculated	No. of successful inoculations
Total	Whole blood		7	5	34	19	10	5	6	4	9	1	8	3	8	0		
	Blood cells				36	19	14	10	24	10	9	5	6	2	4	1	4	0
	Plasma				31	17	10	2	15	4	4	1	7	2				
Per cent of successful inoculations	Whole blood		71.4		55.9		50		66.7		11.1		37.5		0			
	Blood cells				52.8		71.4		41.7		55.6		33.3		25		0	
	Plasma				54.8		20		26.7		25		28.6					

In recent passages, made since about August 14, 1930, the concentration of the transmitting agent has seemed uniformly high in both plasma and suspension of cells of pronounced cases of leucosis, the minimal amount producing leucosis being about 10^{-4} to 10^{-6} cc. The only exception to this is possibly Passage XI F, in which inoculations with 0.15 cc. of whole blood were unsuccessful in four fowls, but three of four fowls injected with 15 cc. of blood died of leucosis. The white blood count of the fowl from which this transfer was made was 1,440,000.

Comment.—The observations are sufficient to exclude the possibility that transfer by plasma is due to a small number of cells contaminating the plasma. Leucemic blood diluted 1:1,000,000 still contains from 100 to 1000 leucocytes per cubic centimeter but does not produce leucosis. (See Table III.) Moreover the concentrated plasma used for inoculation when viewed in the counting chamber appeared cell-free, and nevertheless in high dilution caused leucosis.

The smaller number of successful inoculations with plasma (Table III) as compared with suspensions of cells is best explained in the light of recent observations (10) by assuming that the mode of action of plasma differs from that of cells. The filterable agent in plasma presumably brings about a neoplastic transformation of some of the elements in the bone marrow of the host, whereas leucemic cells are themselves capable of multiplication in susceptible hosts. The concentration of the filterable agent in suspensions of cells and its relation to the cells is worthy of further investigation.

Relation of the Amount of Material Inoculated to the Duration of Incubation Period and of Illness

One would expect that on injecting decreasing amounts of an infective agent the period of incubation will be prolonged and the disease will be milder. To ascertain whether the transmitting agent behaves thus the incubation period and duration of illness of all cases of leucosis recorded in Table III are collected in Table IV.

The duration of illness is actually somewhat longer than the above figures indicate and the incubation period is correspondingly shorter. Although smears were taken from every fowl that appeared ill, in many instances only the routine blood

TABLE IV

Relation of the Amount of Material Inoculated to the Duration of Incubation Period and of Illness

No. of passage	Material injected	Amounts inoculated													
		2 to 15 cc.		0.1 to 1.5 cc.		0.1 to 0.09 cc.		0.001 to 0.009 cc.		0.0001 to 0.0009 cc.		0.00001 to 0.00009 cc.		0.000001 cc.	
		Period of incubation	Duration of illness	Period of incubation	Duration of illness	Period of incubation	Duration of illness	Period of incubation	Duration of illness	Period of incubation	Duration of illness	Period of incubation	Duration of illness	Period of incubation	Duration of illness
		days	days	days	days	days	days	days	days	days	days	days	days	days	days
VI C	Blood cells					57	10								
VII B	Whole blood			16, 16, 22, 131	2, 3, 4, 13										
VII C	Blood cells			75	13	32, 95	7, 44								
	Plasma			59, 110, 117	10, 12, 33										
VII D	Whole blood			29, 39	1, 12										
	Blood cells			30, 30	4, 13			42	35						
	Plasma			29, 30	16, 22			123	8						
VII G	Whole blood			10, 12, 13	1, 1, 5			14, 17	32, 34						
VIII C	Whole blood			17	7	32, 49, 80	1, 7, 20								

VIII E	Whole blood	4, 12, 9, 16, 12, 58	9, 12, 59	3, 11, 13	16	4		35
	Blood cells		10	3	52	3		56
	Plasma		16	1	27	12		
IX B	Blood cells		26, 41, 41	15, 25, 48		28, 40, 41, 76, 66	1, 2, 7, 14	
	Plasma		28, 40, 41, 124	1, 19, 30, 39		1	42, 74	2, 19
X B	Whole blood		29, 29	1, 3				
	Blood cells		30, 45	6, 53	69, 122, 45, 56	1, 10, 1, 3		
	Plasma		30, 30	6, 8	27, 30, 30, 34	7, 23, 37, 44		
X C	Blood cells		48, 49	1, 29	31			
	Plasma		24, 32	1, 3	1			
X D	Whole blood							
XI F	Whole blood	24, 81	6, 7					
XII A	Whole blood		19, 19	4, 8	24, 89	3, 8	52	15
XII B	Blood cells		28	3	27, 27, 32, 98	13, 15, 23, 13		
	Plasma							

TABLE IV—*Concluded*

No. of passage	Material injected	Amounts inoculated													
		2 to 15 cc.		0.1 to 1.5 cc.		0.1 to 0.09 cc.		0.001 to 0.009 cc.		0.0001 to 0.0009 cc.		0.00001 to 0.00009 cc.		0.000001 cc.	
		Period of incubation	Duration of illness	Period of incubation	Duration of illness	Period of incubation	Duration of illness	Period of incubation	Duration of illness	Period of incubation	Duration of illness	Period of incubation	Duration of illness	Period of incubation	Duration of illness
		days	days	days	days	days	days	days	days	days	days	days	days	days	days
XII C	Blood cells Plasma			21	2			37, 98	7, 136						
XII D	Blood cells Plasma			55 40	21 1			13, 54	5, 8					40, 55 81	1, 42 8
XIII D	Whole blood			24, 64	2, 12										
XIII F	Blood cells			17, 19, 19, 29, 50	9, 11, 13, 19, 66			53	75						
XIV B	Blood cells Plasma			26, 37	10, 34	39	13			38, 38	35, 42	56	94		

Compare this table with Table III.

examination made at intervals of 2 to 4 weeks disclosed the onset of leucosis in apparently healthy fowls.

In order to establish the relation of the incubation period to the amount of material injected the inoculations with plasma and with cell suspensions were considered separately in each passage. For example, in Passage VII C (Table IV) the incubation period of leucosis caused by the injections of 0.1 to 1.5 cc. plasma was 29 and 30 days, whereas 0.001 to 0.009 cc. of plasma caused leucosis after an incubation period of 123 days. From this the conclusion is drawn that in this experiment decreasing the amount of plasma injected resulted in lengthening the incubation period.

When the incubation periods shown in Table IV are analyzed in the manner described, the following significant figures are obtained:

Material inoculated	No. of experiments	Effect of decreasing doses on the period of incubation		
		Lengthened	Shortened	No effect
Plasma..............................	7	7	0	0
Blood cells..........................	9	5	1	3
Whole blood.........................	5	3	0	2

Thus the plasma yields results somewhat different from those of suspensions of leucemic cells inasmuch as a decrease in the amounts of plasma regularly increases the period of incubation. A lengthened period of incubation occurred often but not regularly when decreasing amounts of suspensions of cells were injected, in one instance the reverse relation being observed. When the incubation period was prolonged the increase was less with suspensions of cells than with plasma.

Comment.—The mechanism underlying the production of leucosis is complex. Observations already discussed show that transfer by cells is essentially a tumor graft, whereas transfer by cell-free plasma can act only by conferring upon the marrow of the host neoplastic properties. The shortening of the incubation period by increasing the quantity of transmitting agent is like that obtained with a pathogenic microorganism. It is noteworthy, however, that tar, in producing cancer, behaves similarly, a decrease of the amount applied delaying the appearance of cancer.

SUMMARY AND CONCLUSIONS

The concentration of the transmitting agent of leucosis in fowls, as determined by titration, is approximately the same in the suspensions of blood cells and in cell-free plasma; the smallest amount of plasma producing leucosis was 0.000001 cc. and of cell suspension 0.00001 cc. This observation excludes the possibility that transmission of leucosis by plasma is due to the presence of a small number of leucemic cells in the plasma.

The success of inoculations with plasma (20 to 28 per cent of fowls) is, within wide limits, independent of the amount injected (10^{-1} to 10^{-6} cc.). The percentage of successful inoculations with varying quantities of plasma is lower than with corresponding amounts of suspensions of cells (33 to 71 per cent).

When plasma containing the transmitting agent is injected in decreasing amounts the incubation period of the leucosis is conspicuously lengthened. With decreasing amounts of a suspension of leucemic cells the incubation period is not so frequently nor so greatly prolonged.

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STUDIES ON THE NATURE OF THE AGENT TRANSMITTING LEUCOSIS OF FOWLS*

II. Filtration of Leucemic Plasma

By J. FURTH, M.D., AND H. K. MILLER, Ph.D.

With the Assistance of Charles Breedis

(From The Henry Phipps Institute, University of Pennsylvania, Philadelphia)

Recent studies have indicated that the mode of action of leucemic plasma is different from that of leucemic cells. Although inoculum containing leucemic cells (whole blood and cell suspensions) also contains some of the filterable transmitting agent, the leucemic cells are themselves capable of multiplication in susceptible hosts, like cells of transplantable tumors (1). On the other hand, leucemic plasma transmits leucosis only because it contains the agent which causes neoplastic transformation of some of the bone marrow elements of susceptible hosts. This agent is present in the plasma in very high concentration (2). Its properties in general, and particularly its filterability, are best studied with this material, which can easily be obtained free from cells and cellular debris.

Filtration through Silicious Filters

Previous work has already shown that the agent transmitting leucosis may pass Berkefeld filters (3–6). The difficulties of demonstrating filterability were assumed, as with some viruses causing infectious diseases, to be in part due to the association of the agent with particulate matter (cf. 7). In support of this view the observations of Pentimalli (8), may be quoted, who found that the virus of Rous sarcoma is easily adsorbed on susceptible cells. Rous and Murphy (9) assume that the agents transmitting tumors are of relatively large

size as compared with other filterable causes of disease.   They suggest two possibilities to explain the narrow range within which the agents of Rous tumors are filterable: first that they are formed bodies; second that if unformed, they are associated with substances that clog the pores of the filter.   Occasional failures suggested to Gye and Andrews (10) that filterability is not an essential property of the Rous tumors. The evidence presented here on the filterability of the agent points to an explanation somewhat different from those suggested.

It seemed desirable to obtain accurate information as to the quality of the silicious filters that pass or retain the transmitting agent, and to prove that the coarser filters (N and V) retain all cells.   That cells of the Rous tumors may pass coarse Berkefeld filters is maintained by several investigators (11).

Technique.—The filters used were new, of the smallest size manufactured (about 2.5 x 1.5 cm.), and were tested before sterilization.   There are two measurements commonly applied to obtain data suitable for an estimation of the size of the pores of a filter (*cf.* 12).   One of these, the "bubbling pressure," was determined as specified by the manufacturer.   The second, the rapidity of the flow of water through the filter, was measured by us arbitrarily at a negative pressure of 40 cm. Hg after the flow of water had become uniform.   With new filters of the same make the two measurements yielded parallel findings.   Tests of each filter proved particularly desirable with the so called N and V types since they did not show constantly the characteristic differences indicated by their designations.   W filters on the other hand were uniformly finer than either V or N filters.

The material filtered was plasma to which from 20 to 40 per cent heparin solution (1:1000) was added to prevent clotting.   The plasma was further diluted by the addition of broth containing microorganisms, in most instances *Vibrio percolans* (13) and *B. prodigiosus*.   The final dilution of the plasma used for filtration was from 1:2 to 1:3.   In one instance only was it diluted considerably, as suggested by Ellermann.   This material (Passage VIII C) was obtained by adding 1 cc. whole blood to 200 cc. isotonic salt solution and spinning the diluted blood.   Since this experiment was unsuccessful whereas concentrated or only slightly diluted plasma after filtration produced leucosis, high dilutions were not used in subsequent tests.

The filtration was undertaken in most instances at about 400 mm. Hg below the atmospheric pressure.   The negative pressure was somewhat greater in the first four tests (Tables I and II).   The material to be filtered was chilled before filtration, as was the filter itself in several instances.   The filters were readily permeable to the plasma, with the exception of two W filters (Passages XII F and XII H), through which the flow rapidly decreased and filtration was interrupted at the end of 10 to 12 minutes.

From 0.002 to 0.01 cc. of the filtrate was examined in the counting chamber in several experiments, and was invariably found to be free from cells (Table II).

Agar and broth cultures of filtered and unfiltered plasma were kept at 37.5°C. for from 1 to 2 days and subsequently at room temperature for from 2 to 4 days. The cultures of the material to be filtered showed in all instances heavy growth but the filtrates were sterile with two exceptions. *Vibrio percolans* passed one Mandler filter having a bubbling pressure of 320 mm. Hg (Passage XII B) and a Berkefeld N filter with a bubbling pressure of 400 mm. Hg (Passage XII F), but these filters retained *B. prodigiosus*. The failure of coarser filters to retain *Vibrio percolans* was to be expected from the studies of Mudd (13).

Fowls lost through intercurrent disease within the estimated period of incubation have not been included in Table I among the number of fowls injected. The figures given for the periods of incubation and duration of illness are based on examinations of the blood and are therefore approximate but not accurate.

The results of fifteen filtration tests, summarized in Table I, indicate that the success of filtration is less dependent on the porosity of the silicious filter than on such factors as the presence in the plasma of contaminating particles, and substances with affinity for silicious filters.

The agent present in the plasma transmitting leucosis seems to pass fine silicious filters readily if the plasma is free from small particles or substances that obstruct the pores. In two experiments direct filtration through a W filter (Passages XII F and XII H) led within a few minutes to almost complete obstruction and the filtrates were inactive. Filtration through a W filter when preceded by filtration through an N filter (Passage XI G) proceeded rapidly and the filtrate caused leucosis in two of four fowls injected.

The addition of heparin preserves the fluidity of the plasma and therefore facilitates filtration. In the filtration test just described 40 per cent heparin solution (1:1000) was added to the plasma. In the other tests collected in Table I, the plasma contained between 15 and 40 per cent heparin solution. Moreover the precautions usually taken for rapid preparation and handling of plasma in making tissue cultures were adopted to delay clotting.

Repeated centrifugalization at high speed, omitted in the first filtration tests for fear that it might lead to an inactivation of the transmitting agent, seems likewise advisable. Recent experiments have shown that the distribution of the agent in the plasma is not considerably affected by spinning for 2 hours at 3000 R.P.M. In a very

TABLE I

The Results of Filtration of Leucemic Plasma through Silicious Filters

Passage	Filtered plasma						Unfiltered plasma					Suspension of cells or whole blood				
	Type of filter	Amount injected	No. of fowls injected	No. of fowls (Successful inoculation)	Period of incubation (Successful inoculation)	Duration of illness	Amount injected	No. of fowls injected	No. of fowls (Successful inoculation)	Period of incubation (Successful inoculation)	Duration of illness	Amount injected	No. of fowls injected	No. of fowls (Successful inoculation)	Period of incubation (Successful inoculation)	Duration of illness
		cc.			*days*	*days*	*cc.*			*days*	*days*	*cc.*			*days*	*days*
VI C	V	1 to 8	7*	3	102, 139, 139	19, 21, 43						0.05	3	1	57	10
												1.5	2**	0		
VII C	V	0.8	9	2	59, 95	7, 63	0.5	4	3	59, 110, 117	10, 112, 33	0.5	4	1	75	13
							0.01	4	0			0.01	4	2	32, 95	7, 44
							0.0002	4	0			0.0002	4	0		
VIII C	V	0.05	5	0								1.0	6	1	17	7
		0.025	5	0								0.01	5	3	32, 49, 80	1, 7, 20
		0.01	5	0								0.0001	6	0		
VIII E	V	0.5 to 1	8	2*	17, 31	20, 27	0.5	2	1	15	1	0.5	5	4	9, 10, 12, 59	3, 3, 11, 13
							0.005	4	1	27	12	0.005	3	2	16, 52	3, 4
												0.00005	2	0		
X B	N	0.5 to 1	7	5	29, 32, 52 / 52, 59	1, 1, 2 / 39, 41	0.2	3	2	30, 45	6, 53	0.2	4	2	28, 29	3, 1
							0.001	4	2	45, 56	1, 3	0.001	4	2	68, 22	1, 10
							0.000005	4	1	56	35	0.000005	4	0		

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Passage	Material	cc			Fowl nos.		dil.					dil.			Fowl nos.
XI B	N / V	1 / 1	6 / 3*	2 / 0	40, 47	1, 8	1	3	1	45		0.5	6	6	15, 17, 18 / 45, 55, 70 — 5, 7, … / 18, …
XI G	N + W	2 to 4	4*	2	37, 44	1, 2	1	3	2	23, 46	9, 22				
XII B	6 lb. / 9 lb.	1.5 / 1.5	6 / 5	3 / 3	42, 47, 49 / 21, 28, 31	12, 14, 20 / 6, 9, 13	1 / 0.01	2 / 2	1 / 1	28 / 98	3 / 13	1.0 / 0.01 / 0.001	3 / 3 / 1	2 / 3 / 0	19, 19 / 27, 27, 32 — 4, 8 / 13, 15, …
XII C	8 lb.	0.6 to 3	3**	2	61, 101	1, 1	0.5	2	1	21	2	0.1 / 0.001 / 0.00001	3 / 2 / 3	0 / 2 / 0	37, 98 — 7, 13…
XII F	N / W	0.3 to 1 / 0.3 to 1	5 / 3	1 / 0	42	39									
XII H	N / W	0.3 to 3 / 0.4 to 1	3* / 2	1 / 0	100	4	1	3	1	53	1				

* One fowl of each of these groups had mild transient anemia with exception of a fowl in Passage XII H, which had severe anemia. The causation of these anemias by the transmitting agent seemed uncertain and therefore they were grouped as negatives.

** One fowl in each of the two groups indicated had lymphoid leucosis.

successful filtration test, namely XII B, the plasma when inspected in the counting chamber was entirely free from cells before filtration.

TABLE II

Data on the Filtration Tests Given in Table I

Passage	Type of filter	Bub-bling pres-sure	Flow of water per minute	Dura-tion of filtra-tion	Flow of mate-rial filtered	Bacteriological examination of the filtrate
		mm. Hg	*cc.*	*min.*		
VI C	Berkefeld V	—	—	30	Very slow	Sterile
VII C	Berkefeld V	—	—	5	Rapid	Sterile
VIII C	Berkefeld V	—	—	15	Fast	Sterile
VIII E	Berkefeld V	—	—	3	Rapid	Sterile
X B	Berkefeld N	400	—	3	Rapid	Sterile
XI B	Berkefeld N	480	—	4	Rapid	Sterile*
	Berkefeld V	400	—	—	Rapid	Sterile*
XI G	Berkefeld N followed by W	440	52	2	Rapid	—
		770	14	10	Fast	Sterile
XII B	Mandler 6 lb.	320	38	5	Rapid	Slight growth of *V. percolans* but not of *B. prodigiosus**
	Mandler 9 lb.	560	18	3	Rapid	Sterile
XII C	Mandler 8 lb.	430	30	5	Rapid	Sterile*
XII F	Berkefeld N	400	38	7	Fast	Slight growth of *V. percolans* but not of *B. prodigiosus**
	Berkefeld W	760	16	12	Slow, stopped	Sterile*
XII H	Berkefeld N	—	—	—	Rapid	Sterile
	Berkefeld W	—	—	10	Slow, stopped	Sterile

* These filtrates were examined in the counting chamber and were found to be free from cells.

Estimation of the Amount of Transmitting Agent Absorbed by Silicious Filters

A direct determination of the concentration of the agent in the plasma before and after filtration has not been made. Such a determination would require a considerable number of fowls since the figures obtained are significant only if the titration is complete. The percentage of successful inoculations with plasma, as shown in the preceding paper (2), is independent of the infecting dose within the very wide limits of about 10^{-2} to 10^{-6}.

Some intimation of the amount of agent present in the filtrate may be obtained by a comparison of the length of the incubation periods with filtered and with unfiltered plasma (Table III). It has been shown (2) that the incubation period is prolonged when the amount of plasma containing the transmitting agent is decreased. In estimating the effect of filtration upon the incubation period the amounts injected, as shown in Table III, must be taken into consideration. It will then be seen that in three of the nine filtrations the filtrate caused leucosis within about the same period of time as the unfiltered plasma, suggesting that approximately the same amount of the agent was contained in both. In the other six filtration experiments, however, the incubation period was slightly to moderately prolonged, suggesting a slight to moderate decrease in concentration of the agent as a result of filtration (2).

Discussion.—The experiments reported here leave no doubt that the agent transmitting leucosis is filterable through silicious filters. It seems essential that preceding the filtration, the solutions containing the agent be freed as completely as possible from particulate matter. Since the agent is present in high concentration in the plasma, the conditions for obtaining it free from cells and cellular débris are more favorable than with the agents of filterable tumors. Passage through fine filters may be facilitated by preceding filtration through a coarse filter. Similar results were obtained by Krueger and Schultz (14), who found that the virus of poliomyelitis was completely retained by a 2 per cent collodion membrane, but that when filtered first through a 0.5 per cent collodion membrane, it then passed the 2 per cent collodion filters. The mechanism underlying the success of filtrations

through fine filters after preceding filtrations through coarser filters
is not clear *a priori*. Removal of particles larger than the agent
is one possibility but removal of surface-active substances having an

TABLE III

Relation of the Period of Incubation of Leucosis Caused by Inoculations with Unfiltered Plasma and with Plasma Passed through Silicious Filters

Passage	Filtered plasma			Unfiltered plasma			Estimated effect of filtration on the period of incubation
	Amount injected	Period of incubation	Duration of illness	Amount injected	Period of incubation	Duration of illness	
	cc.	days	days	cc.	days	days	
VII C	0.8	77	35	0.5	95	52	No effect
VIII E	0.75	24	24	0.5	15	1	
				0.005	27	12	Moderate delay
X B	0.75	45	17	0.2	38	30	
				0.001	52	2	Moderate delay
				0.000005	56	35	
XI B	1	44	5	1	45	5	No effect
XI G	2	37	2	1	35	16	Slight" delay
	4	44	1				
XII B (6 lb.)	1.5	46	15	1	28	3	Moderate delay
XII B (9 lb.)	1.5	27	9	0.01	98	13	No effect
XII C	0.6	61	1	0.5	21	2	Much delay
	2.5	101	1				
XII H	2	100	4	1	53	1	Much delay

If more than one fowl, inoculated with the same dose, developed leucosis average
figures were given. With regard to inaccuracies in determining the period of
incubation and the duration of illness, see notes to Table IV of the preceding
paper.

affinity for silicious earth may likewise play some rôle. The results
with filtration of the agent of leucosis suggest that under favorable
conditions active agents of tumors may pass the finest silicious
filters.

The view that successful inoculation with filtrates of tumor is due to a few cells that contaminate the filtrate would seem as unfounded as the view that cells may pass silicious filters under ordinary conditions of filtration. The filtrate and in some instances even the plasma used for filtration were free from cells when viewed in the counting chamber. In a few tests, the filtrate was centrifugalized at high speed and the sediment was inspected in the counting chamber but was found to be free from cells. Leucemic plasma, when incubated with embryonic extract at 37.5°C., showed no growth. In one experiment a very coarse (not bacteria-tight) silicious filter, having bubbling pressure of 20 cm. Hg. was used to test filterability of leucocytes in leucemic plasma but none passed through. The passage of phagocytes through living membranes with pores much less than their average size is beyond dispute. This however does not seem to take place under ordinary conditions of filtration through Berkefeld filters. Cooling the filter and material to be filtered may have inhibited active motion of leucocytes.

According to the estimations of Bechhold (15c) the maximum size of pores of W filters is from about 1.5 to 3.5μ and a microbe passes pores of silicious filters if it is from eight to fifteen times smaller than the pores of the filter. On the basis of these figures it may be assumed that the passage of a microbe through a W filter indicates that the microbe is smaller than 0.1 to 0.44μ.

Filtration of Leucemic Plasma through Collodion Membranes of Graded Porosity

Considerable knowledge has been gained recently concerning the technique of ultrafiltration and its application to studies of filterable microbes (cf. Bechhold (15), Elford (16), Krueger and Ritter (17)). Ultrafiltration may serve either to determine the size of viruses or to free them from extraneous matter, but thus far it has not been possible to obtain them in pure form, nor are the values given for their size beyond dispute (cf. 15). Valuable information can however be obtained by filtering a variety of biologically active substances through collodion membranes of graded prorosity under comparable conditions as has been done by Zinsser and Tang (18). The present study was undertaken to compare the filterability of the agent transmitting

leucosis with that of other particulate matter of submicroscopic size.

The passage of organic particles through collodion membranes under conditions determined by us are summarized in Table IV, and tests on the filtration of the agent transmitting leucosis in Table V.

In a series of *preliminary tests* the relation of pressure to the flow of water and to the passage of colloidal substances (Table IV) was determined. Such tests showed the maximum pressure that the various types of filters would resist; *e.g.*, Prussian blue was retained through 1.5 per cent collodion membranes when filtered

TABLE IV

Filtration of Colloidal Substances and Microorganisms through Collodion Membranes

Material filtered	Approximate diameter	Collodion membrane			
		0.5%	1.5%	3%	4.5%
	mμ				
B. *prodigiosus*...........	Above 800	Passed*	Retained	Retained	
Prussian blue............	400	Passed	Retained	Retained	—
Virus of bovine pleuro-pneumonia............	250	Passed*	Retained	Retained	—
Ferric oxide.............	100	Passed	Passed	Passed*	Retained
Arsenic trisulfid.........	90	Passed	Passed	Retained	Retained
Collargol...............	30	Passed	Passed	Retained	Retained
Bacteriophage...........	25	Passed	Passed	Passed*	Retained
Litmus.................	2	Passed	Passed	Passed	Passed
The agent transmitting leucosis.............		Passed	Passed	Passed*	—

* Concentration much decreased.

at a pressure of 4, 8, and 16 cm. Hg but it passed this filter in about one-eighth of the original concentration when the pressure was raised to 32 cm. Hg.   On subsequent lowering of the pressure to 8 cm. Prussian blue continued to pass through the filter in about one-tenth of the original concentration indicating that the membrane had been altered.   The filtrations described here were carried out at a pressure that according to preliminary tests would not injure the collodion membrane.

In all tests the leucemic plasma was obtained by spinning heparinized blood. The blood cells were suspended in Locke solution, shaken, recentrifugalized, and the supernatant liquid was added to the plasma.   The final dilution of the plasma was from 1:2 to 1:3.   The filtration of the agent transmitting leucosis through collodion membranes was carried out in the ice box.

TABLE V

Filtration of Leucemic Plasma through Collodion Filters

No. of passage	Material injected	Amount injected	No. of fowls injected	Successful inoculations		
				No. of successful inoculations	Period of incubation	Duration of sickness
		cc.			days	days
XVIII B	Plasma, unfiltered	0.5	4	1	76	15
	Same, passed through 0.5% collodion membrane	0.5	3	1	6	56
XVII D	Plasma, passed through Berkefeld N filter	0.4	3	1	16	19
	Same, after additional passage through 0.5% and 1.5% collodion membrane	1.5	4	1	25	5
XVII E	Plasma, passed through Berkefeld N filter	0.5	2	0	—	—
	Same, after additional passage through 0.5% collodion membrane	1.25	2	1	23	10
	Same, after additional passage through 1.5% collodion membrane	2.5	2	2	24, 26	19, 86
XIX D	Plasma, passed through Berkefeld V filter	0.5	3	1	24	10
	Same, after additional passage through 0.5% and 1.5% collodion membrane	1.0	3	2	34, 45	7, 9
	Same, after additional passage through 3% collodion membrane	1.5	3	0	—	—
XX D	Plasma, passed through Berkefeld V filter	1.0	3	1	25	5
	Same, after additional passage through 1.5% collodion membrane	1.0	3	2	31, 51	4
	Same, after additional passage through 3% collodion membrane	1.0	3	1	41	11
Total...	Unfiltered					
	Passed through Berkefeld filter	4	1	76	15	
	Passed through 0.5% collodion membrane	11	3	16 to 25	5 to 19	
	Passed through 1.5% collodion membrane	5	2	23 to 61	10 to 56	
	Passed through 3% collodion membrane	12	7	24 to 45	4 to 86	
		6	1	41	11	

The 0.5 per cent *filters* used for preliminary filtration were prepared by us; the denser filters were purchased from Schleicher and Schüll. The filters were placed in a Seitz filtration apparatus supported by meshed wire and several layers of ordinary filter papers. The filters were washed with Locke solution immediately before filtration. The amounts injected are given in terms of the original volume of plasma.

A *bacteriophage* active against *B. coli* was obtained through the courtesy of Dr. F. B. Lynch. The broth culture, after lysis, was centrifugalized, the supernatant fluid passed through a coarse silicious filter and subsequently through a series of collodion filters (Table IV). The size of the bacteriophage is given according to the estimate of Burnet (19).

A culture of the virus of *bovine pleuropneumonia* was obtained through the courtesy of Dr. W. J. Elford. In one experiment the serum broth cultures were filtered directly through collodion membranes. In another test the filtration was made as described for bacteriophage.

Broth cultures of *B. prodigiosus* were diluted with about ten times the volume of Locke solution and passed directly through a 0.5 per cent collodion membrane; part of this filtrate was then filtered through a 1.5 per cent membrane.

The *colloidal substances* were filtered directly through the collodion membranes indicated in Table IV; a preceding filtration through coarse membranes did not improve their filterability through finer membranes.

In Table IV "retained" indicates that the substance filtered could not be demonstrated in the filtrate. However inspection of the lower surface of the membrane, which apparently just retained colored colloidal substances, often showed a discoloration indicating that the material has passed in minute amounts.

Passage XVIII B.—The plasma passed through the 0.5 per cent membrane in quantities of 5 drops per minute at a negative pressure of 12 cm. Hg. One of the fowls injected with unfiltered plasma and given in Table V as negative died of intercurrent disease 33 days after injection. The two fowls injected with filtered plasma and recorded as negatives had transient anemia.

Passage XVII D.—The plasma was not perfectly clear before filtration and its passage through the Berkefeld filter was very slow. This filter had a bubbling pressure of 40 cm. Hg and the flow of water per minute was 40 cc., at a negative pressure of 40 cm. Hg.

Passage XVII E.—The Berkefeld N filter used for preliminary filtration had a bubbling pressure of 41 cm. Hg and the flow of water was 48 cc. per minute. The filtration proceeded rapidly. The final filtration through 1.5 per cent membrane was very slow, about 15 cc. liquid passed the filter in $1\frac{3}{4}$ hours.

Passage XIX D.—The coarse silicious filter had a bubbling pressure of 25 cm. Hg and it permitted 53 cc. water to pass per minute. The filtration proceeded rapidly through the silicious filter, but it was slow through the 0.5 and 1.5 per cent membrane and the 3 per cent membrane was almost impermeable after 10 cc. liquid passed through it in the course of 50 minutes.

Passage XX D.—Preceding filtration the plasma was thoroughly cleared by repeated spinning. The coarse silicious filter used in this test had a bubbling pressure of 22 cm. Hg and the flow of water was 84 cc. per minute. The filtration lasted for 1½ hours and the passage of 10 cc. of liquid through a 3 per cent membrane required 1¼ hours.

Table V shows that the agent transmitting leucosis readily passed 1.5 per cent collodion membranes. Indeed, successive filtrations of plasma through a coarse Berkefeld filter, 0.5 and 1.5 per cent collodion membranes resulted not in a decreased but in an increased activity of the transmitting agent as indicated by the following percentage of successful inoculations: unfiltered plasma 25 per cent (see also Table III of the preceding paper), plasma after filtration through coarse silicious filter 27 per cent, 0.5 per cent collodion membrane filtrate 40 per cent, 1.5 per cent filtrate 58 per cent. These tests permit the conclusion that the agent readily passes 1.5 per cent collodion membranes and suggest the presence of inhibitory substances in leucemic plasma (*cf.* 20, 21). Only one of the six fowls injected with plasma passed through 3 per cent membranes, developed leucosis. This may be interpreted by assuming that the majority of the pores of 3 per cent membranes retain the agent.

DISCUSSION

It is frequently stated that viruses pass collodion filters that retain or barely permit the passage of proteins; it is also asserted that liquid containing viruses may by filtration be obtained free from proteins as determined by chemical tests. The uncertainty of such statements based on qualitative tests becomes obvious from the following considerations.

On filtering colloidal solutions such as ferric oxide or Prussian blue through filters that apparently retain these solutions completely it is not uncommon to find on the further side of the filter minute spots showing the color of the substance filtered. It is evident that the substance has passed the filter in amounts too small to be detected by ordinary tests. Viruses can be detected in very minute amounts mainly because they multiply under favorable conditions. Leucemic plasma may give rise to leucosis in amounts of 0.000001 cc.; it requires much larger amounts of plasma to demonstrate the presence of pro-

teins by ordinary chemical tests. If a filtrate contains less than a certain per cent of a single colloidal substance filtered (*e.g.* 0.1 per cent in the tests of Krueger and Ritter) the filter is arbitrarily designated as impermeable to this matter. Such determinations may be sufficient for a crude separation of colloidal substances or for the determination of the average size of the pores of collodion filters but are inadequate for an estimation of the size of viruses.

These difficulties can be only partly overcome by determining the concentration of the agent before and after filtration because the effectiveness of the agent as shown above may be increased by filtration. Moreover complete obstruction of the smaller pores of the collodion filter may occur leaving only the coarser pores open; through such a filter the passage of liquids is slow, and the agent may not undergo great dilution.

The uncertainty of estimates of the size of viruses on the basis of filtration tests may be illustrated by the following: The agent of Rous tumors was usually retained by the fine silicious filters (W) employed by Rous and from this it would seem larger than the majority of viruses; but Mendelsohn, Clifton, and Lewis (22) found that the agent passed collodion filters which retained proteins. They estimate the size of the agent to be from 15 to 50 $m\mu$.

SUMMARY AND CONCLUSIONS

The agent transmitting leucosis readily passed all types of silicious filters. Filtration is particularly successful when the plasma is freed from particles and substances that would otherwise obstruct the pores of the filter. Filtration through fine filters seems to be facilitated by preceding filtration through coarse filters.

A comparison of the periods of incubation of leucosis produced by unfiltered plasma and plasma passed through silicious filters shows that as a result of filtration, the incubation periods are somewhat prolonged. This suggests a slight or moderate decrease in the concentration of the transmitting agent in the plasma caused by filtration.

Filtration tests through collodion membranes indicate that the agent transmitting leucosis is much smaller than the virus of bovine pleuropneumonia (250 $m\mu$) and that it approximates the size of bacteriophage.

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STUDIES ON THE NATURE OF THE AGENT TRANSMITTING LEUCOSIS OF FOWLS*

III. RESISTANCE TO DESICCATION, TO GLYCERIN, TO FREEZING AND THAWING; SURVIVAL AT ICE BOX AND INCUBATOR TEMPERATURES

BY J. FURTH, M.D.

WITH THE ASSISTANCE OF CLARA B. TAYLOR AND CHARLES BREEDIS

(From The Henry Phipps Institute, University of Pennsylvania, Philadelphia)

Extensive research on tumors due to filterable agents, chiefly by Rous and his associates (1), has disclosed several procedures suitable for the destruction of viable tumor cells without injury to the filterable agents that transmit the tumors. These methods are significant because they prove that transmission can be accomplished by material free from living cells; they are of practical value because they serve to preserve the agents causing tumors for periods of months or years. Prior to investigations in new directions it seemed desirable to ascertain whether the agent transmitting leucosis shares these characteristic properties of the filterable tumors.

The Effect of Desiccation on the Transmitting Agent

In a previous paper (2) an unsuccessful attempt to dry the transmitting agent without inactivation was briefly described. A similar experiment induced Jármai (3) to assume that this agent is different from the agent of Rous sarcoma, which can be dried readily. Rous and Murphy did not succeed in preserving by desiccation all avian tumors that were transmissable by filtrates; they also report that desiccation of Rous' Tumor 1 in many instances resulted in an inactivation of the transmitting agent.

Technique.—Sulfuric acid, calcium chloride and phosphoric anhydride were used for desiccation. The active material, blood cells or whole blood, was placed

TABLE I

Inoculations with Dried Leucemic Blood Cells

No. of passage	Material injected	No. of fowls inoculated	No. of successful inoculations	Notes
VII B	Whole blood, fresh (1.2 cc.)	5	4	
	Whole blood, fresh (0.05 cc.)	3	0	
	Blood cells, 81 days after drying over sulfuric acid	8	0	
XII B	Blood cells, fresh (1 cc.)	3	2	One fowl injected with dried blood died of intercurrent disease 40 days after inoculation
	Blood cells, fresh (0.01 cc.)	3	3	
	Blood cells, 27 days after drying over sulfuric acid	3	0	
XII K	Whole blood, fresh (0.5 cc.)	4	3	
	Blood cells, frozen in liquid air, 33 days after drying over calcium chloride	4	0	
XV B	Whole blood, fresh (0.6 cc.)	4	3	One of the 4 fowls inoculated with blood cells dried over calcium chloride died of trauma 18 days after inoculation
	Blood cells, 27 days after drying over calcium chloride	4	0	
	Blood cells, 27 days after drying over sulfuric acid	3	0	
XVI A	Whole blood, fresh (0.2 cc.)	3	3	(More details of this experiment are given in Table II)
	Blood cells, dried over sulfuric acid	6	5	
	Blood cells, dried over calcium chloride	4	2	
XVII A	Whole blood, fresh (0.1 cc.)	2	2	The fowl successfully inoculated with dried blood cells developed erythroleucosis after an incubation period of about $3\frac{1}{2}$ mos.
	Blood cells, 24 days after drying over phosphorous anhydride	3	1	
XVIII A	Blood cells, fresh (0.1 cc.)	2	2	The fowl successfully inoculated with dried blood cells developed erythroleucosis 42 days after inoculation
	Blood cells, 16 days after drying over sulfuric acid	3	1	
	Blood cells, 98 days after drying over sulfuric acid	3	0	

in Petri dishes in very thin layers and kept in the desiccator *in vacuo* at ice box temperature. The material appeared to be dry within 24 hours. The dry material was stored in the ice box. In some instances it was ground up and dried over again before removal from the desiccator. In one experiment the blood was frozen in an extraction thimble by submersion in liquid air and the frozen blood was placed in a desiccator. When the dried material was gently ground in isotonic salt solution, the resulting suspension was free from visible particles. The amounts of dried blood injected were in all instances somewhat greater than the amounts of fresh blood injected in the control series.

A brief summary of the experiments is given in Table I and a more detailed description of one passage in Table II.

TABLE II

Injections of Leucemic Blood Dried without Inactivation

Passage XVI A

Material injected	No. of fowls inoculated	No. of successful inoculations	Successful inoculations	
			Period of incubation	Duration of illness
			days	*days*
Fresh blood, 0.2 cc....	3	3	12, 13, 19	1, 9, 14
Blood cells, dried over sulfuric acid:				
15 days after drying...	3	3	34, 37, 45	8, 30
54 days after drying...	3	2	35, 37	6, 11
Blood cells, dried over calcium chloride:				
15 days after drying...	1*	1	47	137
24 days after drying...	2	1	43	10

* Two other fowls injected with blood 15 days after drying over calcium chloride died from causes not due to inoculation.

The transmitting agent is shown to have survived desiccation in three of the seven tests recorded in Table I. It is difficult to understand why desiccation as a method of preservation was very successful in Passage XVI A, in two other tests partially successful and in the remaining four unsuccessful. Passage XVI A is fully described in Table II. This experiment leaves no doubt that the agent can be dried without inactivating it; for as soon as the dried blood was found to be active more of it was injected into several fowls, again with success. The incubation period of leucosis produced with blood that had been dry for 15 days and 54 days was about the same, suggesting no mate-

rial decrease in the concentration of the transmitting agent in the dry state within this interval. It is true that the blood injected 15 days after drying caused leucosis in all three fowls injected, whereas the same sample 54 days after drying caused leucosis in only two of the three fowls injected. However, in estimating changes in the concentration of the agent of leucosis, previous experiments (6) have taught us to attach less significance to the percentage of successful inoculations than to the length of incubation period. It is conceivable though that in experiments with dried blood an alteration of the agent may influence the results of inoculation.

In Passage XVIII A, two fowls injected with fresh blood developed leucosis 14 and 16 days after inoculation; the incubation period of leucosis in the fowl injected with dried blood was 42 days. In Passage XVII A both fowls injected with fresh blood developed leucosis, the one after 19 days and the other in 24 days, and the fowl injected with dried blood in about 3½ months. All the fowls showing no symptoms within 4 months after inoculation were killed. It is possible that some of those injected with dry blood would have developed the disease after a longer interval.

Table I shows that all fresh blood used for drying transmitted leucosis. This does not indicate, however, that the fresh blood contained the filterable agent since viable leucemic cells are presumably capable of producing the leucemia (cf. 4). Failure to cause leucosis by injections of dry blood cells may be due either to absence of the filterable agent or to its small quantity or more probably to its inactivation during the procedure of drying. The use of better methods of drying such as those recently recommended by Craigie (5) may lead to more uniform results.

Summary.—Desiccation of leucemic blood without inactivation was successful in three of seven attempts. The experiments suggest that once successfully dried the deterioration of the agent is very slow. The agent can be preserved in a dry state, but the process of drying often results in lessening the transmitting power of the blood or doing away with it completely.

Preservation of the Transmitting Agent in Glycerin

The action of glycerin as well as the process of drying destroys the cells of higher animals whereas most but not all of the filterable agents

causing tumors retain their transmissibility when subjected to either procedure (1). Transmissible sarcoma of the rat is more readily destroyed by concentrated glycerin than by dilute (25 to 50 per cent); but the agents of filterable tumors are best preserved in concentrated glycerin (*cf.* 1).

In Table III experiments are described showing that the agent transmitting leucosis can be preserved in glycerin although in two of three passages the blood became inactive soon after the glycerin was added.

TABLE III

The Survival in Glycerin of the Agent Transmitting Leucosis

Passage	Fresh whole blood					Blood cells preserved in 50 per cent glycerin					
	Amount injected	No. of fowls inoculated	No. of successful inoculations	Incubation period	Duration of illness	Duration of preservation	Amount injected	No. of fowls inoculated	No. of successful inoculations	Incubation period	Duration of illness
	cc.			*days*	*days*	*days*	*cc.*			*days*	*days.*
VII B	1.2	5	4	16 to 131	2 to 13	42	0.2	8	0	—	—
	0.05	3	0	—	—						
XI A	0.5	5	5	12 to 45	1 to 37	50	0.5	4	3	51 to 56	1 to 37
						104	0.5	4	3	35 to 82	7 to 48
XII K	0.5	4	3	33	5 to 34	35	—	3	0	—	—

The material was kept in the refrigerator after the addition of glycerin. Preceding injection it was diluted from five to ten times with Locke's solution. The amount injected is given in terms of the original volume of whole blood.

The donor in the first two experiments (VII B, XI A) had severe myeloid leucemia; that in the third experiment had severe erythroleucosis.

The figures given for the incubation periods and duration of illness are only approximate. They are based on the examination of the blood. The blood smear of one of the fowls injected with leucemic blood preserved in glycerin for 104 days indicated leucosis of about 15 days' duration; subsequent smears during a period of 25 days appeared normal, but microscopically the organs showed erythroleucosis.

Failure to preserve the transmitting agent by the addition of glycerin in Passage VII B may be explicable by the low concentration of the

agent in the fresh blood.   The attempt was likewise unsuccessful to preserve in this manner the blood of a fowl with leucosis produced by inoculations with blood preserved in glycerin (Passage XII K).   The purpose of the latter passage was to isolate a glycerin-resistant strain, but its outcome was unaccountably negative.

The results in Passage XI A show clearly that the agent of leucosis can be preserved in glycerin.

The blood counts of the fowl whose blood was successfully preserved in glycerin were red blood cells 585,000, white blood cells 370,000, hemoglobin 10 (Sahli). The white cells were primitive large mononuclear cells and primitive erythroblasts.   The cells obtained by spinning 20 cc. heparinized blood, were taken up in 15 cc. glycerin solution (glycerin mixed with an equal volume of Locke solution) and were kept in the ice box.

The blood 50 days after the addition of glycerin caused leucosis in three of four fowls injected after an incubation period of from 51 to 56 days, whereas the fresh blood caused leucosis in all five fowls injected after a much shorter interval (from 12 to 45 days).   The prolongation of the incubation period does not necessarily indicate either attenuation of the agent or decrease in its concentration, because the greater activity of the fresh blood may be partly due to the leucemic cells it contains.

As soon as this sample of glycerinated blood was found to be active another group of four chickens were injected with it.   The second inoculation was made 104 days after the addition of glycerin to the blood.

Of the four fowls injected three developed leucosis from 35 to 82 days after inoculation, showing that the activity of the agent did not undergo any appreciable deterioration during the interval of from 50 to 104 days after glycerin was added.

The action of glycerin on the agent transmitting leucosis needs further investigation.   Information as to the rate of deterioration of the agent of leucosis in glycerin can best be obtained by preserving cell-free material containing the agent.   Glycerin added to washed cells for a period of 1 or 2 days may serve as a means of determining the concentration of the agent attached to the cells.   Comparative tests on the action of glycerin on the agent free in the plasma, on the one hand, and on the agent bound to cells, on the other hand, may reveal the rôle played by cells in its preservation in glycerin.

Summary.—The agent transmitting leucosis may be preserved for at least 104 days by the addition of glycerin. The blood preserved in glycerin for this length of time seemed about as active as the same sample tested after 54 days. The incubation periods of leucosis produced by the blood preserved in glycerin were much longer than the incubation periods with corresponding amounts of fresh blood.

The Effect of Freezing and Thawing on the Transmitting Agent

It had been observed in previous experiments that freezing and thawing did not destroy the activity of the transmitting agent (2). The possibility that freezing in ice-salt mixtures was not sufficient to a complete disruption of cells led us to supplement these tests by one in which the cells were submerged in liquid air. On plunging muscle into liquid air there is, according to Smith and Moran (7), no recovery on thawing, however rapidly the cycle of operation is carried out, but dipping leucemic blood in liquid air, as is shown in the following experiment, did not destroy the agent transmitting leucosis.

Cells of leucemic blood were dipped in liquid air and the solidified mass was ground up in a mortar with Celite in the presence of Locke solution. This material was then centrifugalized at 2000 R.P.M., the supernatant liquid removed and re-centrifugalized at the same speed for 10 minutes. This extract when placed in the counting chamber appeared free from cells. It was then injected into the veins of five young chickens, each receiving the amount of extract obtained from about 0.4 cc. of cells. Two of the chickens developed leucosis in from 13 to 29 days after inoculation and the remaining three died of intercurrent disease 20, 32 and 56 days after inoculation. One of the latter had transient anemia; the other two seemed free from lesions suggesting leucosis.

The whole fresh blood used in this experiment was inoculated into four chickens in amounts of 0.2 cc. Three of these developed erythroleucosis from 13 to 22 days after inoculation; the fourth was killed 4½ months after inoculation and appeared healthy.

The modes of action of freezing and of drying are similar according to Smith and Moran (7), the determining factor being the removal of a certain amount of water. Both procedures destroy cells of higher animals but spare the agent transmitting leucosis. Freezing and thawing were in our experiments less injurious to the agent than the process of drying.

Should transmission of leucosis or tumor cells be due to intracellular

enzymes one might expect with Nakahara (8) that freezing would enhance the tumor-producing activity of a suspension of cells, but Nakahara found that freezing and thawing of Rous tumor caused a decrease in its tumor-producing action. Freezing and thawing in our experience likewise decreased the capacity of leucemic blood to transmit this disease. These observations however do not give much information as to the nature of the agent, mainly because leucemic material may reproduce the disease not necessarily by virtue of the free filterable agent it contains but by virtue of the ability of the leucemic cells to proliferate in susceptible animals. The studies of Rous and his associates (9) demonstrated that this is true of tumors due to filterable agents.

Summary.—Freezing of leucemic blood, even by submersion in liquid air does not destroy the transmitting agent.

The Survival of the Transmitting Agent in the Ice Box and in the Incubator

Jármai says (3) that leucemic blood kept in the refrigerator remained active for 10 days; at 37.5° it retained its activity for 13 days; at 56°C. it was inactivated in 30 minutes.

In the experiments described in Tables IV and V the agent when kept at 37.5° lost its activity within 14 days, but it retained some activity for at least 14 days when kept in the refrigerator at 4°C.

Information on the rate of deterioration may be obtained from Table V. This table shows that deterioration is rapid when the blood is kept at 37.5°, but slow when the blood is kept in the ice box. The greater the time in either case the smaller the percentage of successful inoculations and the longer the incubation period.

In the experiment recorded in Table IV the blood cells of a fowl with severe erythroleucosis were separated from the plasma by spinning. The plasma was allowed to clot and the cells were suspended in the corresponding volume of serum. 7 days later the erythrocytes in the sample kept at 37.5°C. had undergone hemolysis but not those in the sample kept in the ice box at 4°C.

The two fowls injected with fresh blood developed leucosis after an incubation period of from 24 to 27 days, and the duration of illness was from 16 to 31 days. One fowl out of ten injected with blood kept in the refrigerator developed leucosis after an incubation period of 41 days and an illness of about 11 days. All ten receiving blood that had been kept at 37.5° remained free from leucosis.

In the experiment recorded in Table V clotting of the plasma was delayed by the addition of 20 per cent heparin solution (1:1000). Samples of blood of a fowl with severe erythroleucosis were sealed with paraffin. Those kept in the refrigerator showed very slight hemolysis after 7 days, moderate hemolysis and slight clotting after 14 days, more pronounced hemolysis and clotting after 21 days. The blood kept in the incubator was clotted and moderately hemolyzed after 7 days; hemolysis seemed complete after 14 days. The clotted samples were cut up with scissors, washed repeatedly with Locke solution; serum and wash liquids were mixed

TABLE IV
Survival of the Agent in the Ice Box and in the Incubator

Whole blood, defibrinated	Immediately after bleeding		Kept at 4°C.		Kept at 37.5°C.	
			For 7 days	For 16 days	For 7 days	For 16 days
Amount injected, cc	0.5	0.005	0.5	0.5	0.5	0.5
No. of fowls inoculated	3	4	5	5	5	5
No. of successful inoculations	2	0	1	0	0	0
No. died of intercurrent disease	1	1	1	1	0	1

TABLE V
Survival of the Agent in the Ice Box and in the Incubator

Heparinized whole blood	Immediately after bleeding	Kept at 4°C.			Kept at 37.5°C.		
		For 7 days	For 14 days	For 21 days	For 3 days	For 7 days	For 14 days
No. of fowls inoculated	3	3	3	3	3	3	3
No. of successful inoculations	3	2	1	0	1	1	0
Successful inoculation:							
Incubation period, days	17, 17, 17	23, 48	33	—	60	40	—
Duration of illness, days	6, 8, 12	13, 28	12	—	Alive	2*	—

* Died of intercurrent disease.

and filtered through a small piece of cotton. The fresh blood was injected in amounts of 0.5 cc., the blood kept in the ice box or in the incubator in amounts corresponding to about 1 cc. of the fresh blood. Table V shows that the agent was still active after 14 days at 4°C., but not for as long as 14 days at 37.5°C.

Survival of filterable agents of Rous sarcoma under the conditions described may be prolonged by the addition of agents that oppose

oxidative changes (*cf.* Pirie and Holmes (10)).   It is probable that the transmitting agent of leucosis behaves in a similar manner.

Summary.—Leucemic blood when kept in the incubator at 37.5°C. lost its capacity to produce leucosis almost completely within 7 days, completely within 14 days.   When kept in the ice box (4°C.) its activity had decreased considerably after 14 days and it became inactive after 21 days.

Attempts to preserve the agent in cultures similar to those described by Maitland (11) and in ordinary tissue cultures have thus far been unsuccessful.

CONCLUSIONS

The filterable agent transmitting leucosis resists drying, retaining its activity for at least 54 days.   The conditions of successful desiccation have not been precisely ascertained.   By the addition of glycerin the agent can be preserved for at least 104 days.   It is not inactivated by freezing in liquid air.   At 37.5°C. it loses its activity within 14 days, but retains some of its activity for at least 14 days when kept at 4°C.

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INFLUENCE OF LIGATION OF PANCREATIC DUCTS O DOGS UPON SERUM AMYLASE CONCENTRATION

By CARL E. JOHNSON, M.D., AND CARL H. WIES

(From the Departments of Surgery and Pathology, School of Medicine, Yale University, New Haven)

Several investigators have undertaken studies which had for their object the estimation of possible changes in amylase concentration in the serum of dogs after the pancreatic ducts had been ligated. A considerable variation is apparent in the interpretation of their results. According to the work of Elman and McCaughan[1] an increase in amylase concentration takes place under these circumstances. The object of the present work is to study such effects with respect to the postoperative interval in contrast with evidence of the effects of all parts of the procedure except that of ligating the pancreatic ducts.

Methods

The method employed for the estimation of amylase concentration has been described by Thompson, Johnson and Hussey.[2] It depends upon the estimation of the time required at constant temperature for a definite change in viscosity to be produced in a mixture of enzyme solution and a starch substrate solution in definite proportion. An indication of the applicability of the method to the present work with sera of dogs is given by the following experiment. Under conditions otherwise the same as in the amylase dilution experiment of the paper mentioned above,[2] the symbolic representation of which will be used in the present communication, three solutions, A, B and C, of dog's serum in 0.85 per cent saline (B and C being successive dilutions to double volume of a portion of A) were employed in the same manner as similarly designated solutions of pancreatin were employed therein. The resulting mean values of T obtained were 0.781, 1.524 and 3.258 and the corresponding values of α', β' and γ' were 0.497, 0.969 and 2.072 respectively. Accordingly, it is evident that the reciprocal

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[1] Elman, R., and McCaughan, J. M., Arch. Int. Med., 1927, 40, 58.
[2] Thompson, W. R., Johnson, C. E., and Hussey, R., J. Gen. Physiol., 1931, 15, 1.

relation between enzyme concentration and T is approximately attained. In the work herein reported the serum amylase concentration is taken as $\frac{1}{T} \cdot D$ where D is the dilution factor.

Serum for this estimation was obtained in the following manner: About 15 ml. of blood was withdrawn from the anterior vein of the dog's fore leg by means of a sterile 20 ml. Luer glass syringe and transferred immediately to a 30 ml. chemically clean sterile test tube. The sterile cotton plug was replaced by a rubber cap so as to prevent evaporation. This was allowed to stand and clot for 20 minutes at room temperature (18–20°C.) and then placed in a refrigerator at 1–2°C. for at least 20 minutes.

The serum was obtained by breaking up the clot with a glass rod and then centrifuging in the usual manner for 15 minutes.

It was found in several different instances that the amylolytic power of sera was markedly diminished after remaining a few hours at room temperature, whereas when such sera were kept in a refrigerator for 4 weeks and then diluted with distilled water in order to compensate for evaporation, estimation of amylase concentration made before and after showed no significant differences. Accordingly, sera which were not used promptly were stored in the refrigerator, but analyses were always made within a 3 day interval thereafter.

<div align="center">EXPERIMENTAL</div>

The animals used in the present experiments were selected and maintained as follows: Young healthy dogs of either sex, weighing from 15 to 20 kilos, were selected. These animals were kept in cages in a separate, well ventilated room, and maintained on a balanced diet as recommended by Cowgill.[3] Then a period of 2 weeks or more was allowed to elapse until their body weight became stable before any observations of amylase concentration were made. Water was available to them at all times.

All blood samples were taken at 8 o'clock in the morning before feeding. One sample per day for at least 4 days preceding the operation was taken to estimate what might be called the normal amylase concentration level for each individual dog.

In preparation for operation the animal was given 1 grain of morphine sulfate subcutaneously ½ hour before the induction of ether anesthesia which was employed in all surgical operations. Aseptic technique was used.

Animals were prepared for operation in pairs. One of these was selected by lot, a midline incision was made through the abdominal wall, the pancreas was thus exposed and the ducts of Wirsung and Santorini and any accessory ducts were doubly ligated with black silk and then severed between these ligatures.

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[3] Cowgill, G. R., *Am. J. Physiol.*, 1928, 85, 45.

The other animal was simultaneously treated in a manner as nearly as possible the same as the first with the exception that these ducts were neither ligated nor severed but instead the pancreas was merely examined. The abdominal incisions were then closed, the animals returned to their cages and maintained as before.

Unfortunately, two of the control animals died within a week after operation. The data are not given for these animals but roughly the same results seemed

TABLE I

Showing the Influence of Pancreatic Duct Ligation upon Serum Amylase Concentration in Dogs

Preoperative	Dog No.					
	1	2	3	4	5	6
days						
3	8	8		12	7	20
2	9	10	7	9	7	11
1	9	9	7	9	6	8
0	7	8	7	11	8	8
Operation.......	Pancreatic duct ligation			Control exposure		
Postoperative						
days						
1						
2	165	131	116	95	10	9
3						12
4	211	98	65	125		
5					16	
6	183		34	131	10	11
7		48				
8	192		34	39	9	11
9		32				
10	116		29	17	9	
11		19				
12	55		19	13		

imminent. Otherwise, postoperatively in each instance, all dogs lost weight for about the first 3 days, after which the control animal quickly regained his normal weight. The dogs whose ducts had been tied off continued to lose weight for several days and refused a considerable amount of their food. Two such animals were kept alive for 10 weeks, having lost approximately one-third of their preoperative weight in that time. Serum samples were taken thereafter for the

estimation of amylase at intervals of approximately 24 hours. The results of estimations made on certain of these are given in Table I and presented graphically in Text-fig. 1.

DISCUSSION

As can be seen in Text-fig. 1, in each instance a rise of several hundred per cent is observed in the amylase concentration of serum of the animals after an operation in which the pancreatic ducts were ligated, the high level being sustained for variable periods of several days
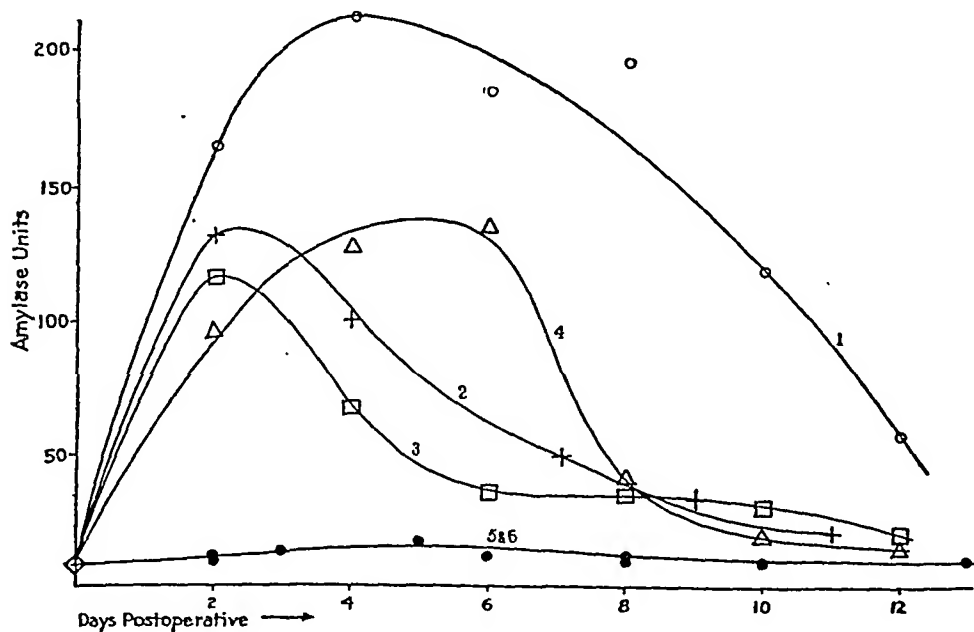
TEXT-FIG. 1

followed by a decline to within at least 100 per cent of the preoperative level, whereas postoperatively the animals in which the pancreatic region was merely exposed no such variation was observed.

Sections taken from the pancreas for microscopic study showed atrophy of the acinar tissue with no obvious changes in the islands of Langerhans, in those animals whose ducts had been tied.

SUMMARY

A sharp contrast has been demonstrated in regard to the postoperative amylase concentration of the sera of dogs, depending upon

whether ligation of the pancreatic ducts was or was not a part of an otherwise prescribed operation. The characteristic feature is a marked rise of several hundred per cent in serum amylase concentration, sustained for several days, which is observed when the pancreatic ducts have been ligated.

AN EXAMINATION OF THE MECHANISM OF PNEUMOCOC-CUS IMMUNITY BY MEANS OF BACTERICIDAL MEASUREMENTS

I. THE REACTION BETWEEN THE ANTICARBOHYDRATE ANTIBODY AND THE PURIFIED SPECIFIC CARBOHYDRATE

BY HUGH K. WARD, M.B.

(From the Department of Bacteriology and Immunology, Harvard University Medical School, Boston)

In a previous communication (1) the author described in some detail the bactericidal effect of defibrinated human blood on the three types of pneumococci and confirmed the work of Sia (2) on the antibactericidal action of the specific carbohydrate. In a second paper (3) the neutralization of this action of the carbohydrate by the corresponding antiserum was studied quantitatively in bactericidal tests. The reaction between the specific carbohydrate and the antiserum has been investigated with great accuracy by Heidelberger and Kendall (4), using chemical methods. This work centered round the specific precipitate between the two reacting substances. The present writer was more interested however in what may be termed the "functional neutralization" of the carbohydrate by the antiserum and this extends far beyond the precipitation zone. For example, a reference to Table I of the second paper (3) mentioned above will show that the Type III carbohydrate has a strong antibactericidal effect in a concentration of 1/75,000, but this effect is specifically neutralized by an antiserum concentration of 1/80,000. It need hardly be said that this concentration of antiserum (1/80,000) would give no visible precipitate with the carbohydrate, indeed the concentration of the antiserum would have to be raised to about 1/150 before the faintest precipitate appeared. The same table shows further that when precipitation does occur with the stronger concentrations of antiserum, the precipitate actually hinders the bactericidal action of the blood. Cromwell and

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Centeno (5) have shown that specific precipitates are ingested by leucocytes and this perhaps interferes with the efficiency of the leucocytes in taking up the pneumococci.

We have not sufficient knowledge at the present time to make any definite assertions about the factors which determine virulence and resistance in the case of the pneumococcus, but the facts strongly suggest: (1) That the capsule is the armour of the virulent pneumococcus, and when this armour is intact, the organism defies phagocytosis. And as far as we know the body can kill the pneumococcus in no other way. (2) That for the virulent pneumococcus the most vital constituent of the capsule is the specific carbohydrate. (3) That the only weapon at the disposal of the body to overcome this carbohydrate defence is the anticarbohydrate antibody (carbohydrate precipitin) which neutralizes the carbohydrate and lays the organism open to phagocytosis. (4) That if there is any free carbohydrate present in the body, it will combine with the anticarbohydrate antibody, leaving so much less free antibody to neutralize the carbohydrate capsules of the living pneumococci.

Since it is possible to measure accurately the carbohydrate-neutralizing power of the anticarbohydrate antibody (3), this theory can be tested to see if it fits quantitatively with the known data of pneumonia serum therapy. But before this is attempted, reference must be made to the recent work of Sabin (6), which has thrown some doubt on the above hypothesis that the anticarbohydrate antibody is the only essential antibody in pneumococcus immunity. Sabin absorbed a Type I pneumococcus antiserum by precipitating the antiserum with Type I carbohydrate. The antiserum after this precipitation gave no further precipitation with the carbohydrate, and Sabin inferred from this that the absorbed antiserum had been deprived of all its anticarbohydrate antibody. However, when this absorbed antiserum was tested on mice, it was found that about 30 per cent of its protective titre was still present. The natural conclusion to be drawn from this experiment, if the premises were correct, was that the protective action of the serum depended mainly on the anticarbohydrate antibody, but partly also on another unknown antibody, which was left untouched by absorbing only with the specific carbohydrate. It seemed inherently unlikely to the present writer that 70 per cent of

the protective power of an antiserum should depend on the presence of one antibody, and that when this antibody was completely removed, the remaining 30 per cent should depend on another antibody. Accordingly the premises were examined more closely. It has been generally assumed that when one can no longer detect precipitation on adding a precipitinogen to an antiserum there is no precipitin left in the serum. If this were the only test for the presence of precipitin, it is self-evident that this would be true, as far as we could tell. But in this particular case the antibody which reacts with the carbohydrate in the form of a precipitate can be detected in another way; *viz.*, by its power to neutralize the antibactericidal effect of the carbohydrate in a bactericidal test. It has already been pointed out in this paper that this neutralization test is far more delicate than the precipitation test. If, then, it can be shown that a carbohydrate-absorbed antiserum, which no longer forms any precipitate with the carbohydrate, is still able to neutralize the antibactericidal effect of the carbohydrate, it is clear that it is incorrect to assume that the anticarbohydrate antibody has been completely removed by absorption. It only shows that the precipitation test is not delicate enough to detect the residuum of antibody. Accordingly an experiment was planned to determine whether the carbohydrate-absorbed antiserum had any neutralizing effect on the carbohydrate.

EXPERIMENTAL

The experiment was carried out with the Type III organism instead of Type I because Type I carbohydrate was not available in sufficient quantity at the time. There is however no reason to believe that the behaviour of Type III antiserum differs from that of Type I antiserum in this respect and indeed former experiments indicated that carbohydrate-absorbed Type I antiserum still had a neutralizing effect on Type I carbohydrate. These Type I protocols are not given here because the complete experiment (the ordinary bactericidal as well as the carbohydrate-neutralizing effect) was not done on the same specimen of absorbed antiserum.

To 3.0 cc. of a strong Type III antiserum were added 2.0 mg. of Type III carbohydrate. The mixture was incubated for 2 hours at 37°C. and was then placed in the ice box for 48 hours. A heavy precipitate had formed and was removed by centrifugation. To the supernatant serum was added 0.1 mg. of the carbohydrate. After incubation for 2 hours and a further 48 hours in the ice box, a very small precipitate had formed. This was removed and the supernatant serum tested for precipitins. It gave no precipitate by the ring test with concentrations of the

carbohydrate ranging from 1/100 to 1/1,000,000. This specimen of serum will be referred to as the absorbed antiserum.

A bactericidal experiment was carried out according to the slightly modified Todd (7) technique described by the present writer in a previous paper (1). To two series of tubes each containing 0.5 cc. of defibrinated human blood were added a certain number of Type III pneumococci (120,000 in this experiment). Decreasing concentrations of the unabsorbed Type III antiserum were added to one series of tubes, and decreasing concentrations of the absorbed antiserum were added to the other series of tubes. The tubes were then sealed, incubated in a rotating machine for 18 hours, the tubes broken open, the contents plated out, and the plates incubated.

TABLE I

No. of Type III diplococci in tube	Concentration of unabsorbed antiserum	Growth	Concentration of absorbed antiserum	Growth
120,000	1/250	+	1/32	0
120,000	1/500	++	1/64	0
120,000	1/1,000	++	1/128	0
120,000	1/2,000	++	1/250	0
120,000	1/4,000	+	1/500	0
120,000	1/8,000	0	1/1,000	0
120,000	1/16,000	0	1/2,000	0
120,000	1/32,000	++++	1/4,000	+++
120,000	1/64,000	++++	1/8,000	++++
120,000	1/128,000	++++	0	++++

Here and in the following tables ++++, +++, ++, + = degrees of growth and 0 = sterility.

Table I shows the result of the bactericidal experiment with the unabsorbed and absorbed antiserum. In the case of the unabsorbed antiserum there is a well marked prozone where the pneumococci are not all killed, but the end-point is clearly seen at a concentration of 1/16,000. In the case of the absorbed antiserum there is no prozone, and the end-point is reached at a concentration of 1/2,000. The prozone is thus seen to be associated with the presence of precipitins in the unabsorbed antiserum. But it is quite obvious that the absorption of the precipitins does not rob the antiserum of all its bactericidal powers, the absorbed antiserum retaining some 12 per cent of its original bactericidal strength. This *in vitro* experiment parallels and confirms Sabin's *in vivo* experiment.

It now remained to test the carbohydrate-neutralizing power of the

absorbed antiserum, and the results of this experiment are seen in Table II.

Every tube contains 0.5 cc. of defibrinated human blood. To one set of these tubes were added a constant amount of the absorbed antiserum, a varying amount of the specific carbohydrate, and a varying number of organisms. The other set of tubes is exactly the same as the first set, save that there was no absorbed antiserum added.

In the last column of Table II is seen the bactericidal effect of the blood alone, and it will be noted that all the concentrations of specific carbohydrate that have been used show an antibactericidal effect in the tubes where there is no absorbed antiserum present. In the tubes where the absorbed antiserum is present, it will be seen that it completely neutralizes the antibactericidal effect of the carbohydrate up to a carbohydrate concentration of 1/15,000.

These experiments indicate, in the writer's opinion, that it is impossible to absorb the whole of the anticarbohydrate antibody out of an antiserum by precipitation with the specific carbohydrate and therefore it is unnecessary to postulate another antibody to account for the fact that such an absorbed antiserum has a definite, though diminished mouse protection titre. This anticarbohydrate antibody appears to account satisfactorily for the bactericidal action of the antiserum in test-tube experiments, for the protective action of antiserum in animal experiments, and is at any rate one of the main factors in determining the crisis in pneumonia.

Turning now to the problem of infection and resistance in pneumonia, it naturally occurred to the writer, as no doubt to many others, that the ultimate outcome in pneumonia might be explained in terms of the specific carbohydrate and the anticarbohydrate antibody in the following manner: If the carbohydrate was still in excess at the time when the patient's vitality was at the critical point, the pneumococci continued to multiply and death was the result. If on the other hand the anticarbohydrate antibody was produced in sufficient quantity to be in excess at this point, the pneumococci were phagocyted, and recovery by crisis was the result. Further, if an amount of antibody which would result in excess were introduced artificially into the circulation, an artificial crisis and recovery should follow.

TABLE II

No. of Type III diplococci in tubes	Concentration of absorbed antiserum										
	1/16	0	1/16	0	1/16	0	1/16	0	1/16	0	0
	Concentration of specific carbohydrate										
	1/5,000	1/5,000	1/15,000	1/15,000	1/45,000	1/45,000	1/135,000	1/135,000	1/400,000	1/400,000	0
1,400,000	++++	+++	0	++++	0	++++	0	++++	0	++	++
140,000	++++	+++	0	++++	0	++++	0	++++	0	++	0
14,000	++++	+++	0	++++	0	++++	0	++++	0	0	0
1,400	++++	+++	0	++++	0	++++	0	++++	0	+	0
140	++	++	0	+++	0	+++	0	+++	0	0	0
14	+	+	0	++	0	++	0	++	0	0	0

TABLE III

No. of Type I diplococci in tubes	Concentration of specific carbohydrate										
	1/1,600	1/1,600	1/1,600	1/1,600	1/1,600	1/1,600	1/1,600	1/2,000	1/4,000	1/1,600	0
	Concentration of antiserum										
	1/16	1/32	1/64	1/128	1/250	1/500	1/1,000	1/1,600	1/1,600	1/1,600	0
400,000	++	+++	++	++	0	0	0	+++	++++	++++	+++
40,000	++	+++	++	0	0	0	0	++++	++++	++++	+++
4,000	++	++	0	0	0	0	0	0	++++	++++	+++
400	+	++	0	0	0	0	0	0	++++	++++	0
40			0	0	0	0	0	0	0	++++	0
4				0	0	0	0	0	0	++++	0

It was quickly seen however that this explanation of infection and resistance in pneumonia was not true. Perhaps the clearest way to demonstrate the inadequacy of such a hypothesis is to examine those cases of Type I pneumonia which die after the injection of 200 cc. of antiserum, a result which is not uncommon if the antiserum is given after the 3rd day of the disease. It is a simple matter to ascertain how much specific carbohydrate would have to be produced in the body so that it would be functionally in excess after the 200 cc. of antiserum had been administered. Table III shows a careful titration of the neutralizing effect of an unconcentrated Type I antiserum on the Type I carbohydrate, the bactericidal technique already described being employed.

In the last column of Table III the bactericidal effect of the blood alone may be observed, and in the preceding column the antibactericidal effect of a 1/1,600 concentration of the carbohydrate. The usual prozone in such experiments is seen with the stronger concentrations of antiserum, then a zone of obvious neutralization, and only when the concentration of antiserum is lowered to 1/4,000 is the 1/1,600 concentration of carbohydrate functionally in excess. In other words there has to be two and a half times more carbohydrate than antiserum if the carbohydrate is to show clearly its antibactericidal effect. Thus if an excess of specific carbohydrate was the critical factor leading to the death of the patient after 200 cc. of antiserum had been injected, there would have been produced in the body $2.5 \times 200 = 500$ gm. of specific carbohydrate. Heidelberger, Sia, and Kendall (8) have shown that when the Type I pneumococcus is grown and allowed to autolyze in broth, 20 litres of this broth contain only about 0.8 gm. of the specific carbohydrate. Accordingly 500 gm. of carbohydrate would be the yield from 12,500 litres of broth. It is obvious that neither 500 gm. of carbohydrate nor anything approaching this amount could be produced in the body. This simple explanation, therefore, of the struggle between the parasite and the host in pneumonia is reduced to an absurdity. Indeed the antibactericidal effect of the specific carbohydrate is so weak when one takes into account the small amount that is found in an autolyzed broth culture of the pneumococcus, that one begins to doubt whether the carbohydrate is an important factor at all. And yet the facts suggest so

strongly that the carbohydrate is associated with virulence, and the antibody, which neutralizes its effect, with resistance, that one hesitates to discount the carbohydrate as a factor in pneumonia, before examining the premises of the theory more closely. This will be done in the second paper of this series (9).

CONCLUSIONS

1. Type III antipneumococcus serum, after absorption with the specific carbohydrate, no longer forms a precipitate with the carbohydrate, but still has a definite, though diminished bactericidal action on virulent pneumococci in a bactericidal test.

2. Such an absorbed antiserum still retains some of its power to neutralize the antibactericidal effect of the specific carbohydrate in a bactericidal test, showing that absorption with the carbohydrate does not remove all the anticarbohydrate antibody from an antiserum.

3. This carbohydrate neutralization test is a very much more delicate method for detecting the anticarbohydrate antibody (precipitin) than the precipitin test.

4. There is therefore no necessity to predicate another antibody to explain the bactericidal action of a carbohydrate-absorbed antiserum, or a similar result in a mouse protection test.

5. The specific carbohydrate has a definite antibactericidal action, but it is demonstrated that, were it present in this form in the body during pneumonia, it could not conceivably be produced in sufficient quantity to influence the disease.

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AN EXAMINATION OF THE MECHANISM OF PNEUMOCOCCUS IMMUNITY BY MEANS OF BACTERICIDAL MEASUREMENTS

II. The Reaction between the Anticarbohydrate Antibody and Type-Specific Products of the Organism

By HUGH K. WARD, M.B.

(From the Department of Bacteriology and Immunology, Harvard University Medical School, Boston)

In the first paper of this series (1), an examination was made of the theory that the outcome in pneumonia largely depended upon (1) the quantity of specific carbohydrate which was produced by the pneumococcus growing and autolyzing in the body and which by its antibactericidal action protected the pneumococcus from phagocytosis; and (2) the quantity of carbohydrate-neutralizing antibody either produced naturally in the body as a result of infection or artificially introduced by injection.

On this theory, excess of the carbohydrate would result in death, excess of the antibody in recovery. When the theory was tested quantitatively, it broke down at once. For it was found that the antibactericidal action of the carbohydrate was so easily neutralized by the specific antibody that the introduction of 200 cc. of antiserum at any stage of the disease would be more than sufficient to neutralize any conceivable amount of carbohydrate that could be present in the body. According to the theory then, no serum-treated patient could possibly die. Unhappily this is not the case.

Up to this point in the investigation, the author had made the natural assumption that the pure carbohydrate (isolated by Avery and Heidelberger (2) in their researches on type specificity) is present as such in the washings of the organisms, in culture fluids, and in the body during pneumonia. And in favour of this assumption is the well known fact that the washings of the organisms, the culture fluids in

which they have grown, and often the urine in the course of the disease contain a substance which precipitates specifically with the antiserum in the same manner as does the purified carbohydrate. This suggests that the substance in the washings of the pneumococcus, in the culture fluids, and in the urine, is identical with the purified carbohydrate, but does not prove it beyond doubt. The possibility remains that the reacting substance in the washings of the pneumococcus, culture fluid, or urine contains the carbohydrate, but is more complex and less stable than the carbohydrate itself. Whether the carbohydrate is isolated from the pneumococci themselves or from the broth in which the organisms have grown and autolyzed, many rather severe chemical manipulations have to be carried out before it can be separated from other substances. If there were a parent substance, which was less stable than the carbohydrate, there would be ample occasion for its breakdown. That a parent substance exists in the pneumococcus itself is suggested by the fact that the carbohydrate is not antigenic, type-specific antibodies being produced only by injection of intact pneumococci or of carbohydrate artificially synthesized with a protein (3). However a pneumococcus autolysate resembles the carbohydrate in that it does not give rise to type-specific antibodies on injection, but again this does not prove beyond doubt that the type-specific pre-cipitinogen in the autolysate and the carbohydrate are indentical. The possibility that the reacting substance in the autolysate is more complex and less stable than the carbohydrate—perhaps a substance intermediate between the antigenic carbohydrate compound in the intact pneumococcus and the carbohydrate itself—was forced on the author as the most likely explanation of some experiments which will now be presented.

When working with a Berkefeld filtrate of a culture of Type III pneumococcus in 0.5 per cent rabbit blood broth, which had been incubated for 5 days, it was found that this filtrate had a strong anti- · bactericidal effect, which was only neutralized by strong concentrations of Type III antiserum in a bactericidal test. This was in marked contrast to the antibactericidal effect of Type III carbohydrate, which in far stronger concentration than could be present in the filtrate is neutralized by a considerably weaker concentration of antiserum. These bactericidal experiments, the results of which are shown in

Tables I and II, were carried out in the manner already described in Paper I (1).

In Tables I and II the last column shows the bactericidal action of the blood alone, and in the immediately preceding columns it will be noted that in the concentrations employed the broth filtrate and the

TABLE I

No. of Type III diplococci in tube	Concentration of Type III broth filtrate						
	1/16	1/16	1/16	1/16	1/16	1/16	0
	Concentration of Type III antiserum						
	1/16	1/160	1/1,600	1/16,000	1/160,000	0	0
800,000	++						
80,000	++	++					
8,000	++	++	++++				
800	+	++	++++	++++	++++	++++	++++
80	0	++	++++	++++	++++	++++	0
8	0	0	++++	++++	++++	++++	0
			++++	++++	++++	++++	0
							0
							0

TABLE II

No. of Type III diplococci in tube	Concentration of Type III carbohydrate						
	1/16,000	1/16,000	1/16,000	1/16,000	1/16,000	1/16,000	0
	Concentration of Type III antiserum						
	1/16	1/160	1/1,600	1/16,000	1/160,000	0	0
400,000	++	++	+				
40,000	++	+	0	++++	++++		
4,000	+	+	0	++++	++++	++++	++++
400	+	++	0	++++	++++	++++	+++
40	0	+	0	++++	++++	++++	0
4	0	+	0	0	++++	++++	0
				0	++++	++++	0
					++++	++++	0

carbohydrate have completely inhibited this bactericidal action. In Table I a prozone with antiserum concentrations of 1/16 and 1/160 is seen. The prozone in these neutralization experiments can always be recognized by the partial growth of the organisms. It is always associated with the formation of a specific precipitate, and if the two

reacting substances are mixed and the precipitate removed before adding the mixture to the blood, a sterile series of tubes results, showing that the prozone is really a zone of neutralization, which is masked by the inhibiting action of the precipitate. The third column of Table I shows that an antiserum concentration of 1/1,600 has no neutralizing effect on the filtrate.

In Table II, a prozone with antiserum concentrations of 1/16 and 1/160 is followed by a series of tubes where an antiserum concentration of 1/1,600 has obviously neutralized the antibactericidal action of the carbohydrate. And a certain amount of neutralization is even seen with a 1/16,000 concentration of the antiserum.

From a comparison of the two tables, it is clear that it requires at least ten times more antiserum to neutralize the broth filtrate than it does to neutralize the carbohydrate. Naturally the first explanation to account for this result would be that the concentration of specific carbohydrate in the broth filtrate is stronger than that used in the experiment shown in Table II. As a matter of fact however, if it is the carbohydrate in the filtrate, titration shows it to be very much weaker. When the broth filtrate is titrated out against the antiserum the end-point is reached at 1/40. A similar titration of the pure carbohydrate gives an end-point of 1/8,000,000. A simple calculation shows that the concentration of the carbohydrate in the filtrate, if it is the pure carbohydrate, cannot be higher than 1/200,000, which means 1/200,000 × 1/16, or 1/3,200,000 in the actual tubes used in the experiment. And this figure has to be compared with the carbohydrate concentration of 1/16,000 used in the experiment shown in Table II. It can, moreover be shown experimentally that a concentration of 1/3,200,000 of the pure carbohydrate has little or no antibactericidal effect on the blood, presumably being neutralized by the natural antibody in the serum.

The second obvious explanation is that the antibactericidal effect is due to a non-specific substance present in the broth filtrate. But this cannot be the case, because the filtrate of Pneumococcus Type III has no antibactericidal effect at all, if Type I or Type II pneumococci are used in the test.

A third explanation is that there is another type-specific antibactericidal substance in the filtrate, which is quite distinct from the

carbohydrate. Enders (4) has recently shown that there is a second type-specific substance in the autolysate of pneumococci, and that this substance has no relation to the carbohydrate. Experiment has shown however (see Table IV) that if a carbohydrate-absorbed antiserum is used in a filtrate neutralization test, such an antiserum is quite definitely weaker in its neutralizing power than the unabsorbed antiserum. This would indicate that the substance in the filtrate which is responsible for the antibactericidal effect is related to the carbohydrate and not to the second type-specific substance, because Enders has demonstrated that the precipitation titre of the antibody against the second type-specific substance is not affected by absorbing antiserum with the specific carbohydrate.

There remains the fourth possibility that the active substance in the filtrate is an intermediate substance between the antigenic carbohydrate complex in the pneumococcus itself and the pure carbohydrate. It is admitted that the only evidence—and that indirect—connecting the filtrate substance with the pure carbohydrate is the above mentioned experiment with the carbohydrate-absorbed antiserum. This hypothetical intermediate substance would be more complex, less stable, and as has been seen, much more difficult to neutralize than the pure carbohydrate. Actually, if a comparison is made on the basis of the precipitinogen content of the filtrate and the pure carbohydrate it requires at least one thousand times as much antiserum to neutralize the antibactericidal effect of the filtrate as it does the same effect of the carbohydrate. The reason for this is quite obscure at present. It may lie in the nature of the union with the antibody, perhaps irreversible in the case of the filtrate substance, and reversible under certain conditions in the case of the carbohydrate. A closely related problem is the explanation of why it is so difficult—or rather impossible—to remove the protective power from an antiserum by absorbing with the specific carbohydrate, and so easy if whole pneumococci are used as the absorbing agent. In both these problems, considerable evidence has already been advanced in these papers that only one antibody is concerned—the anticarbohydrate antibody.

The relation between the active substance in the Type III broth filtrate and Type III pneumonia would be largely theoretical unless it could be shown that a substance with similar characteristics was

present in the lesion of Type III pneumonia.   Through the courtesy of the Boston City Hospital, a pneumonic lung was obtained from an autopsy of a case of Type III pneumonia.   The purulent fluid was expressed from the lung as thoroughly as possible, diluted with an equal part of saline solution, and centrifuged.   The supernatant fluid was passed through a large Berkefeld filter, and the filtrate stored in the ice box.   Table III shows the result of testing the antibactericidal action of the lung filtrate, and also the neutralizing effect of Type III antiserum on this action.   A fairly weak concentration of the lung filtrate is used, because with a stronger concentration, one cannot

TABLE III

No. of Type III diplococci in tube	Concentration of lung filtrate					
	1/320	1/320	1/320	1/320	1/320	0
	Concentration of Type III antiserum					
	1/16	1/160	1/1,600	1/16,000	0	0
600,000	++	+++	++++	++++	++++	++++
60,000	++	+++	++++	++++	++++	0
6,000	++	+++	++++	++++	++++	0
600	++	+++	++++	++++	++++	0
60	+	+++	++++	++++	++++	0
6	0	+++	++++	++++	++++	0

show clearly that the antiserum has any neutralizing effect at all.   The bactericidal technique already described is used.

In the last column of Table III, the bactericidal effect of the blood alone may be observed, and in the preceding column the antibactericidal effect of the lung filtrate.   The partial growth in the first column indicates that the lung filtrate has been neutralized by this concentration of antiserum.   The greater growth in the second column indicates partial neutralization.   But the third and fourth columns show that weaker concentrations of antiserum have no neutralizing effect.   This experiment, then, parallels the one conducted with Type III broth filtrate.

The antibactericidal action of the lung filtrate cannot be due to any non-type-specific substance, because even with a ten times stronger

concentration of lung filtrate, there is no antibactericidal effect if Type I or Type II pneumococci are used in the test. The antibactericidal action of the lung filtrate cannot be due to the presence of pure carbohydrate, because, by precipitinogen titration, it was found that the concentration of carbohydrate, if it were carbohydrate, could be no higher than 1/320,000, and this concentration of pure carbohydrate is neutralized by a weaker concentration of antiserum even than was used in this test.

The neutralizing effect of Type III antiserum which had been absorbed with Type III carbohydrate was then investigated, with the result shown in Table IV. As a control, the effect of the unadsorbed serum was again tested, and also the effect of removing the precipitate

TABLE IV

No. of Type III diplococci in tube	Absorbed antiserum 1/16 — Lung filtrate 1/320	Unabsorbed antiserum 1/16 — Lung filtrate 1/320	Supernatant fluid of mixture of unabsorbed antiserum 1/16 and lung filtrate 1/320	0 — Lung filtrate 1/320	0 — 0
700,000	++++				++++
70,000	++++	++	++	++++	0
7,000	++++	+++	0	++++	0
700	++++	++	0	++++	0
70	++++	++	0	++++	0
7	0	++	0	++++	0
				++++	
				++++	

between the filtrate and the unadsorbed serum before adding the mixture to the tubes. It may be noted here that the specific precipitate formed on adding undiluted antiserum to the undiluted lung filtrate is so bulky that even after centrifugation, it is difficult to obtain any supernatant fluid. No precipitate was however seen on adding the carbohydrate-absorbed antiserum to the lung filtrate.

The third column in Table IV shows clearly that the effect of this concentration of lung filtrate is really neutralized by the 1/16 concentration of antiserum, the partial growth seen in the second column being due to inhibition of the bactericidal power of the blood by the specific precipitate present. The first column shows that when the anticarbohydrate antibody is weakened by absorbing the antiserum

with the carbohydrate, the neutralizing effect on the lung filtrate is very definitely weakened also.   As with the Type III broth filtrate, this evidence points to the active substance in the lung filtrate being a type-specific substance related to, but not identical with the specific carbohydrate.

Assuming that none of the active substance was lost in filtration, it would require about four times the quantity of a strong Type III antiserum to completely neutralize the antibactericidal effect of a given amount of the fluid in this particular lung.   But, although the lungs in this instance contained at the very least half a litre of this fluid, we have no means of ascertaining during life the amount of the concentration of the active substance in the lesion, and therefore it is impossible to say whether the presence of a large amount of this powerful substance at autopsy explains why the Type III antiserum is ineffective therapeutically.   However the behaviour of this substance revives the hope that was dimmed by the examination of the behaviour of the specific carbohydrate—the hope that some day the outcome in pneumonia may be largely explained in terms of a known product of the pneumococcus and a known antibody which neutralizes that product.   The evidence presented here is suggestive that the antibactericidal substance in the Type III broth filtrate and the Type III lung filtrate is the sought for product of the pneumococcus, but the evidence is not strong enough to justify a more definite claim than that.

While these experiments were in progress, Dr. Sutliff of the Boston City Hospital kindly supplied a specimen of defibrinated blood from a convalescent case of Type III pneumonia, the blood being drawn about 5 or 6 days after the crisis.   It was thought that the action of convalescent blood on the specific carbohydrate, on the broth filtrate, and on the lung filtrate might throw some light on the natural mechanism of recovery and perhaps give a hint as to the method to be attempted in serum therapy.   Table V shows the result of this experiment.   The same technique was employed with the convalescent blood as with the normal blood.

In Table V, the last column shows that the convalescent blood is strongly bactericidal, and the third and fourth columns demonstrate that the blood was able in addition to neutralize a considerable con-

centration of Type III carbohydrate. The complete sterility indicates that the concentration of precipitin (anticarbohydrate antibody) was weak, because had it been strong, a precipitate would have formed on the addition of the carbohydrate, and all the organisms would not have been killed—see Table II. Yet despite this weakness in precipitin content, the first and second columns of Table V show that the convalescent blood was able to neutralize the lung filtrate fairly well, and the broth filtrate very well. The only way that it is possible to parallel these results with a mixture of normal blood and antiserum is to use a much stronger concentration of antibody (well within the precipitation zone) and remove the precipitate between the antibody and the lung filtrate (or broth filtrate) before adding these substances to the blood—see third column, Table IV.

TABLE V

No. of Type III diplococci in tube	Type III lung filtrate 1/320	Type III broth filtrate 1/16	Type III carbohydrate 1/16,000	Type III carbohydrate 1/160,000	
600,000	++++	+	0	0	
60,000	+++	0	0	0	0
6,000	0	0	0	0	0
600	0	0	0	0	0
60	0	0	0	0	0
6	0	0	0	0	0

The action of the convalescent blood on these filtrates is difficult to explain. It is weak in precipitin (anticarbohydrate antibody) but neutralizes the filtrates, whereas a similar concentration of precipitin in an artificial mixture of normal blood and dilute antiserum (see Column 3 in Tables I and III) has no neutralizing action. This would point to the presence of another unknown antibody in the convalescent blood, were it not that in stronger concentration the antiserum has a neutralizing antibody for the filtrates, which is presumably the anti-carbohydrate antibody, since its neutralizing action is weakened by absorbing the antiserum with the carbohydrate. The problem is complicated and puzzling as it is left in this stage, but it is possible that the clue to Type III serum therapy lies hidden in the maze. The tentative hypothesis upon which the author is working at present is

that the neutralizing antibody present in the convalescent blood differs in some subtle way from the neutralizing antibody present in the antiserum.   As an explanation, this is admittedly weak and unsatisfactory.   However, it must be borne in mind that the stimulus to the production of antibodies is different in the two cases.   In the artificially produced antiserum, the stimulus is first dead pneumococci and later living pneumococci, which are no doubt soon killed after they are injected into the horse.   In the actual disease the stimulus is the organism growing, multiplying, and later autolyzing in the lesion. Obviously the first point to determine is whether the active substance in the lung filtrate is antigenic.   All previous experience is against this possibility, type-specific antibodies being apparently produced only by intact pneumococci.   Nevertheless this question is being investigated once more, although with no great optimism, and so far with no success.

Two other specimens of Type III convalescent blood neutralized the Type III broth filtrate in a similar manner.

A few experiments have been carried out with Type I pneumococcus. The filtrate of a 5 day broth culture of the Type I organism behaves more like the Type I carbohydrate, its antibactericidal action being neutralized by the weaker concentrations of antiserum. Several attempts have been made to grow the organism in fresh undiluted human serum.   It did not always grow very well, but whenever there was good growth the filtrate had a strong antibactericidal effect, and like the Type III lung filtrate was not neutralized by the weaker concentrations of antiserum.   Up to the present, no filtrate from a lung of an untreated Type I pneumonia case has been available for testing.

DISCUSSION

Despite the great progress that has been made during the last decade in our knowledge of the pneumococcus, the mechanism of infection and resistance is not yet clearly understood.   In any theory of immunity, proof or disproof must of course in the end rest on animal experimentation, but this method of investigation has disadvantages in attempting an analysis of the mechanism of immunity, because the various factors cannot be separated or their quantitative relationship studied, save with the greatest difficulty.   It is in this field of analysis

that the bactericidal technique can be used with advantage, if its obvious limitations are borne in mind.

One can hardly doubt that the problem of virulence and resistance in the case of the pneumococcus centers round the specific carbohydrate and its specific antibody. · Accordingly it is very desirable to understand how these substances react with one another in pneumococcal infection, and in defibrinated human blood the conditions are fairly similar, though of course not identical with those that pertain in the living body. It has been possible to demonstrate by this method the following facts.

1. That a serum which gives a negative precipitin test (using the specific carbohydrate as the precipitinogen) may still have a considerable antibody content against the specific carbohydrate. Accordingly it is unjustifiable to assume that an animal or human being has no type-specific immunity against a type pneumococcus because no specific precipitins can be demonstrated in the serum.

2. That the antibactericidal action of the specific carbohydrate, though definite, is seen to be very weak, when the small amount of the polysaccharide that is present in an autolyzed culture is taken into account. If the actual carbohydrate is present in the body in pneumonia, it is difficult to see how there could possibly be sufficient to influence the course of the disease.

3. That in the filtrate of an autolyzed broth culture of Type III pneumococcus, and more significant still, in the filtrate of a lung obtained at autopsy from a patient dead of Type III pneumonia, there could be demonstrated a type-specific substance which has a far more powerful antibactericidal action than the purified carbohydrate. This substance appears to be related to the specific carbohydrate, and its nature and possible influence on the disease have been discussed in the text.

4. That as far as the evidence from three specimens of convalescent blood goes—and that is admittedly not far—the blood just after crisis appears to be more efficient in neutralizing the autolysate than a corresponding mixture of normal blood and antiserum. If this can be confirmed, it may eventually throw some light on the problem of serum therapy. No adequate explanation has yet been offered of why serum therapy is only effective in producing a crisis in the early stages

of Types I and II pneumonia, is ineffective at any stage in Type III pneumonia, while the natural crisis occurs much later in the disease.

In attacking the baffling problem of pneumococcal immunity by investigating the functions of the pneumococcal products and the neutralizing action of the antiserum on these products, one is but doing in pneumonia what was done many years ago in diphtheria. The details in the two problems are very different, but broadly speaking the products of each organism are its defences, as the respective antiserums are the main defences of the host. Unfortunately we cannot attack the toxemia of pneumonia directly as we can in diphtheria, but the experiments with the lung filtrate indicate that the products of the pneumococcus have to be reckoned with, even though they are not themselves toxic to the body cells.

CONCLUSIONS

1. There is in the filtrate of a 5 day broth culture of Type III pneumococcus a type-specific substance which has a very powerful antibactericidal action. If the precipitinogen content of the broth filtrate and the specific carbohydrate is taken as the basis of comparison, it requires approximately one thousand times as much antiserum to neutralize the broth filtrate as is necessary to neutralize the specific carbohydrate. The active substance in the broth filtrate appears to be related to the specific carbohydrate. Its possible nature is discussed.

· 2. A similar substance, but in stronger concentration, was found in the filtrate of a lung from a Type III pneumonia autopsy. The influence of this substance on the disease is discussed.

3. One specimen of Type III convalescent blood, though comparatively weak in anticarbohydrate antibody (precipitin) was better able to neutralize the broth filtrate and the lung filtrate than a corresponding mixture of normal blood and antiserum. Two other specimens of Type II convalescent blood neutralized the Type III broth filtrate efficiently.

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THE FURTHER SEPARATION OF TYPES AMONG THE PNEUMOCOCCI HITHERTO INCLUDED IN GROUP IV AND THE DEVELOPMENT OF THERAPEUTIC ANTISERA FOR THESE TYPES*

By GEORGIA COOPER, CAROLYN ROSENSTEIN, ANNABEL WALTER, AND LENORE PEIZER

(*From the Bureau of Laboratories of the Department of Health of the City of New York, New York*)

An investigation of the possibility of treating cases of pneumonia with specific antisera other than Type I and Type II antisera was undertaken because clinical results indicated that pneumonia cases were benefited slightly, if at all, by treatment with heterologous antisera. For this purpose it was necessary to classify further the infecting types of pneumococci, to prepare diagnostic antisera for their identification and specific therapeutic antisera for treatment.

The results given in an earlier report (1) are as follows: 58 per cent of pneumococcus strains isolated from lobar pneumonia cases in adults which did not fall into Types I, II and III were classified in ten types which were termed Types IV to XIII. The cross-protective power of potent antisera for Types I, II and III against these types was tested by protection tests in mice and was found to be very low. Monovalent antisera of high agglutinative and high protective value were prepared for these types by injecting rabbits. Also, monovalent antisera suitable for clinical trial were prepared for five types by immunizing horses. A study of the possibility of preparing suitable polyvalent antisera was begun and there was an indication that the potency of the polyvalent antiserum for the separate types injected as compared with good monovalent antisera was lowered somewhat in proportion to the number of type strains injected.

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The results obtained and the problems encountered in the further development of the investigation with the exception of the possibility of preparing suitable polyvalent antisera are discussed in this report. The findings in regard to production of polyvalent antisera will be given in a separate report.

Further Separation of Types and Correlation of Types with Severity of Disease

As 42 per cent of the Group IV strains from the lobar pneumonia cases of adults were left unclassified and several of the strains were isolated from the blood stream or from the sputa of severe or fatal cases it seemed worth while to carry further the classification of strains. Antisera were prepared for strains isolated from the more severe cases. The strains from the first series of cases which were not used for serum production were discarded on account of the difficulty of maintaining a large number of stock cultures intact through the summer months. A classification of strains freshly isolated from cases in the season 1928 and 1929 was carried out. Strains isolated from cases of pneumonia of children were also studied. In the season 1929 and 1930 a new series of cultures was studied. In 1931 only strains which were not classifiable or gave doubtful reactions or were wanted for special work were investigated. The majority of strains as in the earlier work were isolated from cases at the Harlem and Bellevue Hospitals.[1]

In this study nineteen additional types were identified which were termed Types XIV to XXXII, continuing the numbering from the Types I, II and III of the early investigators and the types called IV to XIII by us.

These types were correlated as far as we were able with the types described by others. The similarities of the whole classification are as follows:—

Type IV—Pn. 10, Griffith (2); Group IV B, Robinson (3).
Type V—Sub. II A, Avery (4); Group IV E, Robinson (3).

Type VI—Sub. II B, Avery (4).
Type VIII—Group IV A, Robinson (3); Atypical III, Sugg, Gaspari, Fleming and Neill (5).
Type XV—Pn. 98, Griffith (2).
Type XXI—Pn. 160, Griffith (2).
Type XXII—Pn. 41, Griffith (2).

The cross-agglutination reactions of representative strains of each type with antisera for each of the types were studied. The antisera of rabbits or horses or of both were used for these tests.

The cross-agglutination and cross-protection reactions of Types II and V and of Types III and VIII have been discussed in the earlier report (1).

A number of strains which were agglutinated slightly to moderately in Type VI (Sub. II B of Avery) antisera, were found to be agglutinated to titer in an antiserum prepared for one of the strains. This antiserum agglutinated Type VI cultures to a marked degree. The strains corresponding to the original Type VI strains, we have termed Type VI a. The strains agglutinating to titer in the new antiserum and slightly to moderately in the original Type VI antiserum we have termed Type VI b. At first we called the latter strains Type XXVI; but later changed the terminology; because, on account of their cross-reactions with Type VI, division of the strains into two types did not seem to be justified. After further investigation, it may be found that the differences are negligible from a practical point of view and that by the use of a dominant strain for serum production, they can be disregarded. Then the strains can be classified simply as Type VI.

Cross-protection tests were carried out with Type VI a and Type VI b antisera. Two Type VI b antisera, one having 200 and the other 500 units, each had 100 units protection against Type VI a; Type VI a antisera having 500 units had 200 to 500 units against Type VI b. On account of these strong cross-reactions it would seem to be feasible to group the strains of VI a and VI b together for serum treatment.

Also, there were cross-agglutination reactions between Types VII and XVIII, especially strong with the horse antisera and with the Type VII strains which had been selected for immunizing horses. The cross-reactions with rabbit antisera prepared for representatives

of both types were less.   Cross-protection tests were carried out with horse and rabbit antisera for these types.   The Type XVIII horse antiserum had 500 protection units both against a Type VII culture and against the homologous strain.   One Type XVIII rabbit antiserum had 20 units of protection against VII and 50 units against Type XVIII; another had 5 units against VII and 1000 units against Type XVIII.   A Type VII horse antiserum had 5 protection units against XVIII and 200 units against Type VII.   Type VII antisera from rabbits had less than 1 unit of protection against Type XVIII and 200 units against Type VII.   The fact that the Type VII strain used for protection tests was considerably less virulent for mice than the Type XVIII strain may account for the discrepancy in the degree of cross-agglutination and cross-protection.   We have tested many strains of Type VII and Type XVIII for their cross-agglutination reactions and found them to differ considerably.

There were moderate cross-agglutination reactions between Types XV and XXX.   We have not been able to carry out satisfactory cross-protection tests because of a lack of fully virulent test strains for these types.

The strains examined in this study were isolated from sputa, throat cultures, blood, spinal fluid and postmortem cultures.   All the spinal fluid and blood cultures examined yielded only one serological type. A few of the mouse heart cultures were found to contain two types. The findings in such cases with the type identification made at the hospital is as follows: "Type III?" contained Types III and XVIII; "Type XIX," Types III and XIX; "Type IV," Types IV and XVIII; "unclassified," Types XIII and XVIII; "unclassified," Types IV and XXII; and "unclassified," Types XI and XIV.   In only one of these cases was it clearly evident which type was the more infective, *i.e.* the case in which Types IV and XVIII were found in the culture made from the heart blood of the mouse inoculated with sputum; Type XVIII was isolated from the blood, pleural fluid and spinal fluid.   It is probable that in cases in which more than one type of pneumococcus is found, examination of blood taken during convalescence for antibodies for the different types would give helpful information.

A classification of 181 strains isolated from lobar pneumonia in adults in the season 1928 and 1929 was carried out by agglutination

tests with Antisera IV to XX and of 97 strains isolated in the season of 1929 and 1930 with Antisera IV to XXIX. In the 1929–30 cases special attention was given to the presence of more than one type of pneumococcus. The separation of the strains into types, the percentage of all strains in each type and the number and percentage of deaths are given in Table I. Types IV, V, VII and VIII were most prevalent, constituting 50 per cent of the total. There was an increase in Type VI *b* cases in the late winter and spring of 1931. This occurred after we had discontinued examination of all strains of the different types and therefore we have no figures as to the relative number of cases caused by this type in that period. In this series of cases pneumococci and *B. friedlaenderi* were found in three cases, pneumococci and tubercle bacilli in one, and two types of pneumococci in eight. The organisms were found early in the disease and it is impossible to tell in the majority of the cases, from the data available, whether one or both organisms were responsible for the infections.

Sixty-eight strains isolated from lobar pneumonia in children in the season of 1928 and 1929 and sixty-eight strains isolated in the season 1929 and 1930 were studied (Table II). Types V, VI *a* and XIV were found most often and amounted to 39 per cent of the total. Two or more types were found in thirteen of the lobar pneumonia cases. Types VI *a* and XXIII occurred most often in such combinations. The rôle of the different types when more than one are present needs investigating. The case mortality of 12 per cent (19 per cent in children under 2 years and 6 per cent in the older group) was low in comparison with the adult series.

In their studies of the bacteriology of pneumonia in children, Plummer, Raia and Schultz (6), using our antisera reported that 50 per cent of the strains were classifiable in Types I to XIII; Types I and VI were found most frequently, Type I occurring in 9.5 per cent of the cases and Type VI in 12.9 per cent. In the later study (7), about 85 per cent of the strains were classifiable in Types I to XXIV and Types I, IV, VI, VII, XIV and XVIII were most prevalent. Also, in order to test the reliability of the original type determination, strains isolated from different sources in the same child, as throat (swab), chest fluid, blood, sputum, ear and organs postmortem, were examined. There were a few disagreements; but the findings, in general, confirmed the validity of the original typing.

TABLE I

Grouping of Strains of Pneumococci of So Called Group IV from Lobar Pneumonia in Adults into Specific Types
(Seasons 1928–29, 1929–30)

Types	Total No. of strains	Percentage of all strains in each type	No. of deaths	Percentage of deaths in each type	13–40 years		Over 40 years		Age unknown	
					No. of strains	No. of deaths	No. of strains	No. of deaths	No. of strains	No. of deaths
IV	38*[1,2,3,4,5]	14	12[1,5]	32	27[1,2,3,4,5]	6[1,5]	6	4	5	2
V	39	14	14	36	26[4]	8	13	6		0
VI a	11	4	2	18	7	0	4	2		
VI b	5[5]	2	2	40	3[5]	1	2	1		
VII	36[6]	13	10	28	29[6]	5	7	5		
VIII	27	10	7	26	18[7]	4[7]	8	3	1	0
IX	10[8]	4	6[8]	60	7	3	3[8]	3[8]		
X	8[9]	3	2[9]	25	3	0	4[9]	2[9]	1	0
XI	6	2	2	33	2	0	2	0	2	2
XII	8[3]	3	2	25	5[3]	1	1	1	2	0
XIII	10[10]	4	3	30	5	2	3	1	2[10]	0
XIV	5	2	2	40	2	0	2	1	1	1
XV	4[2]	1	1		4[2]	1	0			
XVI	2	1	1		2	1	0			
XVII	5	2	2	40	2	1	1	1	2	0
XVIII	12[6,10]	4	6[5]	50	7[5]	4[5]	3	1	2[10]	1
XIX	6[7]	2	2[7]	33	3[7]	1[7]	3	1		
XX	7	3	2	29	5	1	0		2	1
XXI	2	1	1		1	0	1	1		1

XXII	2[4]	1	0	2[4]		0	0			
XXIII	2[11]	1	1[11]	1		0	1[11]	1[11]		
XXIV	1[12]	0.5	1[12]	0		0	1[12]	1[12]		
XXV	3	1	1	1		0	2	1		
XXVII	0	0		0		0	0			
XXVIII	1	0.5	0	1		0	0			
XXIX	0	0		0		3	0			
Negative	28†	10	6	22	21	5	3	1	3	0
Total......	278		86	185	32	42	70	37	23	7
	(271 cases)			(179 cases)			(70 cases)		(22 cases)	

* The small figures indicate cases in which two or more organisms were found which might have been the cause of the pneumonias. Cases in which two types of pneumococci were isolated are listed under both types.

[1] Type IV and B. friedlaenderi.   [2] Types IV and XV.   [3] Types IV and XII.   [4] Types IV and XXII.   [5] Types IV and XVIII. Type XVIII in blood, spinal fluid and postmortem lung culture.   IV and XVIII in sputum.   [6] Types VII and VI b.   [7] Types VIII and XIX.   Type XIX in blood and postmortem lung.   [8] Types IX and III.   [9] Type X and B. friedlaenderi. B. friedlaenderi in blood, X and B. friedlaenderi in sputum.   [10] Types XIII and XVIII.   [11] Type XXIII and tubercle bacillus.   [12] Type XXIV and B. friedlaenderi. B. friedlaenderi in blood. XXIV and B. friedlaenderi in sputum.

† Twenty cultures isolated season 1928–29 were negative in Antisera I to XX. Antisera for the other types were not available. One culture was negative in Antisera I to XXIII, one negative I to XXIV, two negative I to XXVII and four negative I to XXIX. The cultures which were not completely examined were discarded or were lost before the work was completed.

TABLE II

Grouping of Strains of Pneumococci of So Called Group IV from Lobar and Bronchial Pneumonia in Children into Specific Types

(Seasons 1928–29, 1929–30)

Types	Total No. of strains	Percentage of all strains in each type	Lobar pneumonia — No. of deaths	Percentage of deaths in each type	Lobar — Less than 2 yrs. No. of strains	Lobar — Less than 2 yrs. No. of deaths	Lobar — 2-13 yrs. No. of strains	Lobar — 2-13 yrs. No. of deaths	Bronchial — Less than 2 yrs. No. of strains	Bronchial — Less than 2 yrs. No. of deaths	Bronchial — 2-13 yrs. No. of strains	Bronchial — 2-13 yrs. No. of deaths
IV	6	4	1	17	3	1	3	0	0	0	2[14]	0
V	16	12	2	13	1	0	15	2	1	1[15]	0	0
VI a	20*[1,2,3,4,5,6]	15	2[6]	10	11[2,3,6]	2[6]	9[1,4,5]	0	6[16]	1	3[14]	1
VI b	1	1	0		1	0	0		1	1	0	
VII	6[4,7]	4	0	0	1[7]	0	5[4]	0	1	1	2	0
VIII	3[5]	2	1		3[6]	1[6]	2[5]	0	0		0	0
IX	5[6]	4	1[6]	20	1[8]		1	0	0		0	0
X	2[8]	1	1		1		1	1	0		1[15]	
XI	5	4	0	0	2[9]		4	0	1	0	0	
XII	2[9]	1	0		0		0	0	1	0	0	
XIII	1[5]	1	0		11	3	1[5]	0	0		2[16]	
XIV	17[10]	13	3	18	1		6[10]	0	1	1	0	
XV	7[11]	5	0	0	1		6[11]	0	0		0	
XVI	2	1	0		1		1		1		0	
XVII	4	3	0		1		3		2	1	2[17]	1
XVIII	5[2,12]	4	0	0	4[2,12]		1		2	1	0	
XIX	7	5	0	0	4		3		4	1	0	
XX	3[3]	2	1		2[3]	1	1		1	0	0	
XXI	1	1	0		1		0		1	1	0	

	Total											
XXII	2[13]			0		1[12]	1				1	
XXIII	5[7,9,10,13]	1	1	1		2[7,9]	3[10,13]	1	1		1	
XXIV	1	4		0		0	1	0			0	
XXV	0	1		0		0	0				0	
XXVII	0			0		0	0				0	
XXVIII	0			0		0	0				0	
XXIX	2			2		1	1				0	0
Negative	13†	10	3	20	17	9	4	3	23	9	0	1
Totals.....	136 (126 cases)	15	12	23	17	63 (57 cases)	73 (69 cases)	4	23	9	14 (12 cases)	3

* The small figures indicate cases in which two or more pneumococcus types were found which might have been the cause of the pneumonias. Such cases are listed under each type. [1] Types VI a and I. [2] Types VI a and VII. [3] Types III, VI a and XX. Type III in blood. [4] Types III, III VI a and VII. [5] Types VI a, VIII and XVIII. [6] Types VI a and IX. [7] Types VII and XXIII. [8] Types I, III XVIII and III. [9] Types XII and XXIII. [10] Types I, XIV and XXIII. [11] Types II and XV. [12] Types XVIII and XIV. [13] Types I and XXIII. [14] Types IV and VI a. [15] Types VI a and III. [16] Types XI and XIV. [17] Types

† Eleven cultures isolated season 1928–29 were negative in Antisera I to XX. Antisera for the other types were not available.

TABLE II

Grouping of Strains of Pneumococci of So Called Group IV from Lobar and Bronchial Pneumonia in Children into Specific Types

(Seasons 1928–29, 1929–30)

Types	Total No. of strains	Lobar pneumonia							Bronchial pneumonia			
		Percentage of all strains in each type	No. of deaths	Percentage of deaths in each type	Less than 2 yrs		2–13 yrs		Less than 2 yrs		2–13 yrs	
					No. of strains	No. of deaths	No. of strains	No. of deaths	No. of strains	No. of deaths	No. of strains	No. of deaths
IV	6	4	1	17	3	1	3	0	0		2[14]	0
V	16	12	2	13	1	0	15	2	1	0	0	0
VI a	20*[1,2,3,4,5,6]	15	2[6]	10	11[2,3,6]	2[6]	9[1,4,5]	0	6[15]	1[15]	3[14]	1
VI b	1	1	0		1	0	0		1	1	0	
VII	6[4,7]	4	0	0	1[7]	0	5[4]	0	1	1	2	
VIII	3[5]	2	1		1	1	2[5]	0	0		0	
IX	5[6]	4	1[6]	20	3[6]	1[6]	1	0	0		0	
X	2[8]	1	1		1[8]	0	1	1	0		1[16]	0
XI	5	4	0	0	1	0	4	0	1	0	0	0
XII	2[9]	1	0		2[9]	0	0		1	0	0	
XIII	1[5]	1	0		0		1[5]	0	0		2[16]	
XIV	17[10]	13	3	18	11	3	6[10]	0	1	1	0	0
XV	7[11]	5	0	0	1	0	6[11]	0	0		0	
XVI	2	1	0		1	0	1	0	0		0	
XVII	4	3	0		1	0	3	0	0		2[17]	
XVIII	5[2,12]	4	0	0	4[2,12]	0	1	0	2	1	0	1
XIX	7	5	0	0	4	0	3	0	4	1	0	
XX	3[3]	2	1		2[3]	1	1	0	1	0	0	
XXI	1	1	0		1	0	0		1	1	0	

XXII	2[12]	1	0	0	1[12]	0	1	0	0	1	1	0
XXIII	5[7,9,10,13]	4	1	1	2[7,9]	0	3[10,13]	1	1	1	1	1
XXIV	1	1	0	0	0	0	1	0	0	0	0	0
XXV	0	0	0	0	0	0	0	0	0	0	0	0
XXVII	0	0	0	0	0	0	0	0	0	0	0	0
XXVIII	0	0	0	0	1	0	1	0	0	0	0	0
XXIX	2	0	0	20	9	0	4	0	2	1	0	0
Negative	13†	10	3	3	9	3	4	0	2	1	0	1
Totals....	136	15	23	12	63	17	73	4	23	9	14	3
	(126 cases)				(57 cases)		(69 cases)				(12 cases)	

* The small figures indicate cases in which two or more pneumococcus types were found which might have been the cause of the pneumonias. Such cases are listed under each type. 1 Types VI a and I. 2 Types VI a and VII. 3 Types III, VI a and XX. Type III in blood. 4 Types III, VI a and X. 5 Types VI a, VIII and XIII. 6 Types VI a and IX. 7 Types VII and XXIII. 8 Types I, III and XXII. 9 Types XII and XXIII. 10 Types I, XIV and XXIII. 11 Types II and III. 12 Types XVIII and XIV. 13 Types I and XXIII. 14 Types IV and VI a. 15 Types VI a and III. 16 Types XI and XIV. 17 Types XVIII and III. Antisera for the other types were not available.

† Eleven cultures isolated season 1928–29 were negative in Antisera I to XX.

In the course of our work, examinations were made of a few strains from normal individuals and from respiratory conditions other than pneumonia. The distribution of those which could be classified is

TABLE III

Grouping of Strains of Pneumococci of So Called Group IV from Normal Individuals, from Respiratory Cases Other than Pneumonia and from Pneumococcus Meningitis

Types	From normal individuals	From respiratory cases other than pneumonia	From pneumococcus meningitis
IV	2	1	1
V	4	1	0
VI *a*	9	4	0
VI *b*	0	1	1
VII	1	1	2
VIII	5	1	2
IX	1	1	0
X	1	0	1
XI	3	1	1
XII	0	0	2
XIII	2	2	0
XIV	0	1	1
XV	1	1	0
XVI	1	2	0
XVII	1	0	0
XVIII	2	1	4
XIX	8	4	0
XX	0	3	2
XXI	0	3	1
XXII	0	0	0
XXIII	1	3	1
XXIV	1	3	1
XXV	0	1	0
XXVII	1	2	1
XXVIII	0	0	0
XXIX	0	1	0
XXX	0	1	0
XXXI	0	1	0
XXXII	0	0	0

given in Table III. Types VI *a* and XIX were most prevalent. About 30 per cent of the strains were unclassifiable in the antisera which were available.

Webster and Hughes (8), employing antisera for Types I to XXV in a study of the incidence and spread of pneumococcus types in normal persons, found Types III, XIII and XIV most prevalent.

Plummer, Raia and Schultz (6), testing with antisera for Types I to XIII, found Type VI most prevalent and Type III next in number, in children who did not have pneumonia.

Dr. Griffith[2] in England, found representatives of the majority of the types in the healthy population of a boys' school.

Dr. Wilson G. Smillie[2] studied the incidence of pneumococcus types in St. John, Virgin Islands, and with antisera for Types I to XVIII found the majority of these types present there.

These results show that the pneumococcus types found in lobar pneumonia cases may be found in normal individuals and that the types found in New York are also present in distant localities. Types III, VI *a*, XIII, XIV and XIX, during the period of these studies, apparently were more prevalent in normal individuals in this locality.

The classification of strains from pneumococcus meningitis also is given in Table III. Fourteen of the recently separated types were found. Type XVIII, found in four cases, outnumbered the others.

Preparation of Diagnostic Antisera

Antisera for determination of types have been prepared in quantity for nearly all the representative strains by injection of horses.[3] It is our plan to have sufficient antisera available for carrying out the identification of types in hospital laboratories by ordinary routine methods. Also, we plan to preserve representative strains of all types over a considerable period of time and to furnish these strains and small amounts of homologous antisera to investigators who are studying the incidence of pneumococcus types in other localities, in order that the general distribution of the types may be determined and the classifications of other workers may be correlated with ours. As we pointed out in our earlier paper, Group IV strains isolated from various conditions had been studied by different workers; but, with few exceptions, strains of the types separated by them could not be ob-

[2] Personal communication.

[3] The immunization of horses for pneumococcus antisera was carried out by Dr. G. W. Welton and Dr. E. M. Schryver at the New York City Antitoxin Laboratories at Orisville, New York.

tained.   We believe it is very desirable to preserve a set of represent-
ative type strains.

Preparation of Therapeutic Antisera

Therapeutic antisera for fourteen types have been prepared for
clinical trial.

The potency of these was calculated in units per cc. on the basis that a unit
is ten times the smallest amount of antiserum that protects a majority of mice
against approximately 100,000 fatal doses of culture.   As soon as expedient, a
lot of antiserum for each type was carefully titrated and adopted as a standard.
All preparations were then compared with the standard antiserum which was
included as a control in each set of protection titrations.   It is apparent that
antisera for different types, having equal values in units as calculated by this
method, are not necessarily of equal therapeutic value in human cases; because
the test cultures for the types differ in their virulence for mice and the virulence
of pneumococci of different types for mice does not correspond to their virulence
for human beings.   Estimations in units are of value because they permit com-
parison of the relative potency of different antisera for the same type.   The
comparative value of the antisera for different types for treatment of pneumonias
of human beings can only be found by clinical trial.

Laboratory tests made in an earlier study (1) indicated that thera-
peutic antisera for Types I and II had very little value in infections
caused by heterologous types.   These antisera, having 1000 to 2000
protective units for the types injected, usually had less than 1 unit
against heterologous types as calculated by tests in mice.   The highest
cross-protection was by a Type II serum against Type V, amounting
to about 20 units.   In our recent work, with antisera for some of the
new types, we have encountered instances of greater cross-protection;
for example, Types XVIII and VII discussed above.   The early work
with Type I indicated that therapeutic antisera for that type should
have at least a certain minimum potency (0.2 cc. of antiserum should
protect against 0.1 cc. of highly virulent culture), which is equivalent
to about 50 to 100 units per cc. as we now calculate the strength.   We
have found that antisera for Type I having 1000 units or more can be
obtained in certain horses after immunization for 8 months or longer.
It is probable that many of the antiserum preparations which were
used in the early clinical trial were several times, perhaps even ten
to twenty times, the minimum strength.   It is our belief that the

stronger antisera are more efficacious than the weaker product because the necessary protective units can be given in smaller volume and consequently less foreign proteins are injected. Also, in severe cases more antibodies can be given in a dose than would be possible with

TABLE IV

The Development of Protecting Antibodies in Horses in Response to Injections of Types IV, V, VI a, VII, VIII, XVIII and XXII, and Types I and II

Types	Horse	Protection in units per cc. after immunization for the following mos.														
		2	4	6	8	10	12	14	16	18	20	22	24	26	28	30
IV	174*		100	100	100	100										
	297*, †	5	20	50	100	500	500	1000		100	50					
	321*		100	100	200	100	100	100	200	500	1000	1000				
V	188*		75	200	500	1000	500	500								
VI a	258			100	200	200	200	200	200	500	1000	1000				
VII	241*		500	500	200											
	282*	100	200	500	200											
	485	100	200	200	200											
VIII	292*	40	100	200	500											
	373	−20	100	100	200	200	100	100	100	100						
XVIII	235	20	50	200	500	200	500	500	750	500	500	500	500	500	500	
XXII	372*		200	500	500	1000	1000									
I	121*	−50	500	500	1000	1000	1000	1000	1000	1000	750	500	300	300	500	
	170*	100	200	200	500	500	100	500	500	200						
	265*	−20	100	200	500	500	500	500								
	54	20	50	200	500	1000	500	500	500	500	500	500	1000			
II	50*	50	100	100	100	100	200	200	200	500	500	500	500	200	500	1000
	99‡		50	50	50	50	75	75	75	200	200	200	200	200	500	750
	222*		50	100	100	100	200	200	500							
	223*		100	100	100	200	200	200	200	200						

* Horse died within 2 months of the last bleeding reported.

† Horse 297 was ill for several months.

‡ Horse 99 produced antisera having an average of 500 units per cc. in the period from 30 to 48 months after immunization.

low grade antiserum. At present we have selected 500 units as the minimum strength for our Type I and II preparations. We are trying to produce antisera for the other pneumococcus types which by laboratory tests will be equal to high grade Type I antiserum.

Moderate amounts of therapeutic antisera have been prepared for

Types IV, V, VI *a* and *b*, VII, VIII, IX, X, XIII, XIV, XV, XVII, XVIII, XX and XXII. These antisera were usually concentrated[4] in order that as potent products as possible could be made from the moderate number of bleedings available.

The stimulation of antibodies in horses inoculated with Types IV to XXII was similar in general to that with Types I and II as far as could be judged by agglutination, precipitation and protection tests (Table IV). For some types, antisera were as easily prepared as for Type I; for others, there was more difficulty as for Type II; but none of those studied apparently offer as great difficulty as Type III. Where we have sufficient data, the potency of the antisera produced for each type is given in the summary of results with each type.

Summary of Results with Each Type

The data collected for each type as to prevalence, severity of cases and the production of antisera during the whole period of the study are summarized below.

We have described the virulence of the strains as fully, very highly, highly, moderately, slightly virulent and non-virulent. "Fully" indicates that 1 to 5 diplococci, as determined by colony count in poured plates, killed mice in 3 days or less; "very highly" indicates that 1 to 50 diplococci killed in 4 days or less; "highly" indicates that 50 to 500 diplococci killed in 4 days or less; "moderately" that 500 to 500,000 killed in less than 4 days; "slightly" that 500,000 to 5,000,000 were required to kill.

Type IV (Pn. 10, Griffith; Group IV B, Robinson).—Fifty-seven strains from lobar pneumonia of adults were studied. Thirty-four cases from which this type was isolated were rated as severe, twenty-three as moderate or mild. Nineteen patients died, of whom nine were shown to have positive blood cultures. Three patients shown to have positive blood cultures recovered. One patient developed a Type IV meningitis. This type was found in six cases of lobar pneumonia in children. Five cases were moderate in severity. One child who was shown to have a positive blood culture died. Type IV was one of the more prevalent types in adults and less prevalent in children.

Examination of further Type IV strains supported our earlier observation that they are generally fully virulent for mice. There was no difficulty in maintaining

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[4] The concentration of the antisera was carried out under the direction of Dr. L. D. Felton at the Harvard Medical School or Dr. E. J. Banzhaf at the New York City Research Laboratory.

the virulence of the strain used in determining the potency of antisera by mouse protection tests.

Antisera for Type IV prepared in horses averaged between 100 and 200 units per cc.; after immunization for 1½ years, the antiserum of one horse had 500 units. Concentrated preparations made from early bleedings had 800 to 1200 units per cc. It is possible that the potency of these antisera, as estimated by protection tests in mice, is uniformly low because of the high virulence of the test strains for mice. The agglutination and precipitation titers, however, were not especially high.

The polysaccharides of Type IV have been studied by Heidelberger and Kendall (9). They found a type-specific carbohydrate which was different from those of Types I, II and III, a carbohydrate which was chemically similar but was inactive in specific reactions and a species-specific polysaccharide "C" substance found also in Types I, II and III by Tillett, Goebel and Avery (10).

Type V (Sub. II A, Avery; Group IV E, Robinson).—Forty-five strains from lobar pneumonia of adults were studied. Thirty-one cases were severe and fourteen were mild. Nineteen patients died, including fourteen who were shown to have positive blood cultures. Five patients from whom positive blood cultures were obtained, recovered. This type was found in sixteen cases of lobar pneumonia of children. Thirteen cases were moderate in severity; three were severe. Two children died of whom one was shown to have a positive blood culture. Type V was one of the more prevalent types in the pneumonias of both adults and children.

This type was found to be the probable causative agent of an epidemic of colds, bronchitis and pneumonia in a children's home (11).

Type V strains when grown in blood broth had a greater tendency to hemolyze blood cells than the other types studied. A special avidity for blood cells may account for the fact that there was a large percentage of positive blood cultures in Type V cases.

After immunization of horses for 8 months antisera were obtained which had 500 to 1000 units per cc. The agglutination and precipitation titers of the sera also were high. When the antiserum was concentrated ten times using methods which were successful with the antisera of the majority of the other types, the loss was great; the finished preparation having only 500 to 1000 units. The development of a method for concentrating this type antisera which will ensure a good recovery is needed.

Strains of this type were moderately virulent for mice. The maintenance of the test strain in a fully virulent condition required very frequent mouse passage. Because of the marked cross-reactions of many Type V strains with Type II antisera and *vice versa*, it would be desirable, in order to avoid mistakes, where serum treatment is to be given to cases of either type or a careful study is to be made of their prevalence and prognosis, to titrate all strains reacting with either antiserum with antisera for both types. Perhaps it will be advisable to have the

antiserum preparations for treatment potent for both types. We are undertaking the preparation of such antiserum. It will take a long while to collect sufficient statistics to make a comparison of the relative therapeutic value of any of these new antisera and Type I antiserum. We have received favorable case reports however, that indicate Type V antiserum is probably of value.

Type VI a (Sub. II B, Avery).—This type was found in eighteen cases of lobar pneumonia of adults. Five were rated as severe, thirteen as mild. Three of four patients who died were shown to have positive blood cultures. This type was found in twenty cases of lobar pneumonia in children. Five cases were severe; fifteen were moderate. Two children died. Type VI *a* was frequently found also in normal individuals and in respiratory conditions other than pneumonia.

Type VI *a* strains were generally slightly to moderately virulent for mice. Frequent mouse passage was required to keep the test culture fully virulent.

The antiserum produced from a horse which was immunized during a period of nearly 2 years was comparatively low in value having only 100 to 200 units. The agglutination and precipitation titers were correspondingly low. Concentrated serum preparations were obtained which had 800 to 1000 units per cc. The immunization of another horse has been more successful, the antiserum of this horse having 500 units after immunization for 8 months and 1000 units after a year and a half. The difference in the response of these two horses to immunization with Type VI is noteworthy. We have encountered similar difference, however, in horses immunized with Types I and II. The reason is not apparent. It is not because of an inherent inability to produce antibodies, for several of the horses which have not responded well to immunization with pneumococci have produced potent antiserum when inoculated with other organisms.

Type VI b.—Data were obtained on eight cases of lobar pneumonia in adults. Five cases were rated as severe and three as moderate. Four patients died, two of whom were shown to have positive blood cultures. This type was found in the spinal fluid of a case of meningitis following a fractured skull. A case in a baby 9 months old was moderate in severity.

The strains were generally moderately virulent for mice.

On account of the strong cross-reactions of Types VI *a* and VI *b*, a Type VI *a* horse was given injections of both types. This horse whose serum had a potency of 500 units against Type VI *a* and 100 units against Type VI *b*, after 3 months immunization with both types had 500 units per cc. and after 6 months had 1000 units per cc. against each type.

Type VII.—Type VII was found in forty-seven cases of lobar pneumonia in adults. Twenty-six cases were rated as severe, nineteen as moderate. Twelve patients died of whom five were shown to have the organism in the blood stream. Two patients shown to have positive blood cultures recovered. Type VII was isolated from two spinal fluids sent to us for examination. This type was found in six lobar pneumonias of children; one case was severe, the others moderate or mild. This type was prevalent in the pneumonias of adults but rare in those of children.

Antisera having 500 to 1000 units were obtained from horses after 6 to 8 months immunization. We are not sure whether it was by chance or because of a susceptibility of horses to inoculation with this strain that there were many deaths of horses being immunized with this type. The method of concentrating antisera of this type as of Type V requires further investigation. Only 500 to 1000 units per cc. of protective antibody were recovered in preparations in which the serum was concentrated ten times.

Strains of this type usually were moderately virulent for mice. The test strain required very frequent passages to maintain full virulence.

Reports of the serum treatment of cases caused by this type have been encouraging.

Type VIII (Group IV A, Robinson; Atypical III, Sugg, Gaspari, Fleming and Neill).—This type was isolated from thirty-one cases of lobar pneumonia in adults. Twelve cases were rated as severe, sixteen as moderate; eight patients died. Five of seven patients which were shown to have positive blood cultures died. Two pneumonia cases developed meningitis and Type VIII was found in the spinal fluids. This type was found in three cases of lobar pneumonia in children; two were mild; one child who was shown to have a positive blood culture, died.

Long periods of immunization were required to obtain antiserum of suitable potency for this type. Antisera having 100 to 300 units per cc. were obtained from two horses after they had been immunized for 8 months. Concentrated antisera were prepared having 800 to 1500 units per cc.

The strains of this type generally were fully virulent for mice. No special care was necessary to maintain the virulence of the test strain.

There are marked cross-reactions between this type and Type III. Differences in colony morphology, however, clearly differentiate strains of this type from freshly isolated Type III strains. We have never found Type VIII to have the large mucoid colonies characteristic of Type III. If agglutination and precipitation reactions are chiefly relied upon for identification of Type III strains, Type VIII may be confused with it. We believe that titrations of strains reacting with either type should be made with both antisera.

Type IX.—Type IX was isolated from sixteen cases of lobar pneumonia in adults. Seven were rated as severe, nine as moderate. Seven patients died including two which were shown to have positive blood cultures. One patient shown to have a positive blood culture recovered. This type was isolated from three cases of lobar pneumonia in children; two cases were moderate in severity; one child died.

Antisera having 200 units per cc. were prepared in one horse. Concentrated antisera having 1000 units per cc. were prepared from this antisera.

Strains of this type generally were only slightly virulent for mice.

A suitable test strain was discovered only after prolonged search.

Type X.—This type was isolated from thirteen cases of lobar pneumonia of adults of which two were rated as severe, three as moderate. Six patients died,

two of these were shown to have positive blood cultures. One patient having a positive blood culture recovered. Type X was isolated from the spinal fluid of one meningitis case sent to us for examination. This type was found in two cases of lobar pneumonia in children; one case was mild and the other terminated fatally.

The strains were of low virulence for mice.

Type XI.—This type was isolated from ten adult cases, of which four were severe and six moderate. Three patients died. One case from which a positive blood culture was obtained was rated as moderate in severity. This type was isolated from five lobar pneumonia cases in children; three cases were moderate in severity; two were severe; of the latter, one terminated fatally.

Antisera were prepared having 200 to 500 units per cc.

The strains were moderately to fully virulent for mice.

Type XII.—This type was found in twelve cases of lobar pneumonia in adults; six were rated as severe and six as moderate. Four patients died; of whom three were shown to have positive blood cultures. Type XII was found in two spinal fluids sent for examination. This type was isolated from two severe cases of lobar pneumonia in children.

The strains were generally of low virulence for mice.

Type XIII.—This type was isolated from fourteen cases of lobar pneumonia of adults. Seven were rated as severe and seven as moderate. Three patients died; two of these were shown to have positive blood cultures. This type was isolated from one child having a mild lobar pneumonia.

Type XIII was found by Webster (8) to be one of the most prevalent types in the normal individuals which he studied.

The strains which we examined were slightly to moderately virulent for mice.

Type XIV.—This type which was one of the most prevalent types in the pneumonias of children was found in only five pneumonia cases of adults. Three were severe and two mild. Two patients died, of whom one was shown to have a positive blood culture. Type XIV was found in one spinal fluid sent for examination. This type was isolated from nineteen cases of lobar pneumonia in children. Seven cases were rated as severe and twelve as moderate or mild. Four children died; of whom three were shown to have positive blood cultures.

The strains of this type were moderately virulent for mice.

Type XV (Pn. 98, Griffith).—This type was found in only four cases of lobar pneumonia of adults, two were severe and two moderate. One patient who was shown to have a positive blood culture died. This type was found in seven cases of lobar pneumonia in children; six were mild and one was severe.

With one exception the few strains which we had of this type were slightly virulent for mice.

Type XVI.—This type was found very infrequently. It was found in two adult cases; one patient having a positive blood culture died; the other case was mild. It was found in two lobar pneumonias of children.

The few strains studied were slightly virulent for mice.

Type XVII.—Strains were obtained from six cases of which four were severe and two moderate. Two patients died, of whom one was shown to have a positive blood culture. This type was found in four lobar pneumonia cases in children; one was severe, the others were mild.

The strains were slightly to moderately virulent for mice.

Type XVIII.—Strains from twelve cases of lobar pneumonia in adults were studied. Ten cases were severe and two moderate. Six patients, all of whom were shown to have positive blood cultures, died. One of the above patients developed meningitis and Type XVIII was isolated from the spinal fluid. This type was isolated from four spinal fluids sent to us for diagnosis. It was found in five lobar pneumonia cases in children; one was severe; the others were moderate or mild.

Type XVIII strains were moderately to fully virulent for mice. The virulence of our test strains has been maintained without difficulty.

The one horse immunized with this type produced antiserum having 500 to 1000 units per cc. Because of the cross-reactions between Types VII and XVIII this horse was transferred to inoculations of both types and after a few months had 1000 units per cc. against Type VII and 500 units per cc. against Type XVIII. Concentrated refined antiserum having 5000 units against Type XVIII was prepared from the monovalent antiserum.

Type XIX.—Type XIX was isolated from six cases of lobar pneumonia of adults of which five were severe and one moderate. One patient shown to have the organism in the blood died. This type was isolated from seven lobar pneumonia cases in children; three cases were severe; four were moderate or mild. It was found moderately prevalent in normal individuals.

The strains were moderately virulent for mice.

Type XX.—This type was isolated from seven cases of lobar pneumonia in adults. Three were rated as severe and two as moderate. Two patients shown to have positive blood cultures recovered. This type was found in three lobar pneumonia cases in children; two were severe and one was mild. One child died.

The strains of this type differed markedly in their virulence for mice, the majority of the strains being moderately virulent.

Type XXI (Pn. 160, Griffith).—Three strains of this type from lobar pneumonia of adults were studied. Two cases were rated as severe and one as moderate. Two patients died, of these one was shown to have the organisms in the blood and spinal fluid. This type was isolated from one mild lobar pneumonia in a child.

The strains of this type were generally slightly virulent for mice.

Type XXII (Pn. 41, Griffith).—This type was isolated from two mild cases of lobar pneumonia of adults. It was found in two lobar pneumonia cases in children; one was severe and the other mild. Griffith,[2] in England, found this type in a number of normal individuals.

An antiserum having 500 units per cc. was prepared after immunization of a horse for 6 months.

Strains of this type generally were very highly virulent for mice but tended to lose their virulence rapidly.

Type XXIII.—Seven strains of this type from lobar pneumonia of adults were studied. Six of the cases were severe and one moderate. Two patients died, one of whom was shown to have positive blood culture. One patient who had a positive blood culture recovered. This type was isolated from five cases in children; two were mild and three severe; one of the latter terminated fatally. It was found also in the spinal fluid of a child who developed posttraumatic meningitis.

The strains of this type were generally slightly to moderately virulent for mice.

Type XXIV.—This type was isolated from five moderately severe lobar pneumonias of adults and from one fatal case that had *B. friedlaendcri* in the blood. It was isolated also from a spinal fluid sent to us for examination.

Griffith,[2] in England, found this type in a moderate number of normal individuals.

Strains of this type were moderately to fully virulent for mice.

Type XXV.—Five strains from the lobar pneumonia of adults were studied. Two patients died, both of whom were shown to have positive blood cultures. Three cases were rated as moderate.

Strains of this type were generally very highly virulent for mice.

Type XXVII.—This type was isolated from two moderately severe cases of lobar pneumonia in adults. It was also found in a spinal fluid sent for diagnosis and in a case of follicular tonsilitis and a case of measles.

The few strains examined were moderately to very highly virulent for mice.

Type XXVIII.—Four cases of lobar pneumonia in adults were moderate in severity.

The strains examined were very different in their virulence for mice, ranging from slightly virulent to fully virulent.

Type XXIX.—We have data on three adults, one died and two cases were severe. This type was found in three mild cases of lobar pneumonias of children.

The strains were very irregular in their virulence for mice, the majority however being highly virulent.

Type XXX.—We have data on three mild cases in children.

Three strains were examined for their virulence for mice; one was highly virulent, one moderately virulent and the other non-virulent.

Type XXXI.—We have data on two cases in adults from which this type was isolated; one had lobar pneumonia of moderate severity, the other died.

The strains examined were moderately virulent for mice.

Type XXXII.—We have not been able to get data on cases from which this type was isolated.

The strains were moderately virulent for mice.

DISCUSSION

The difference in the incidence of cases and in the relative severity of the infection caused by the different types indicates that all the types are not of equal importance, and it is questionable whether it is practical to attempt to produce therapeutic antisera for those which are less prevalent and less virulent. Although there is no immediate benefit to individual patients in determining the type where no therapeutic serum is available, it seems worth while to continue examination for all the types and maintain a full supply of diagnostic antisera until it is learned whether there is a seasonal and periodical variation in the types in this locality and what their distribution in other localities is. The statistics which have been collected by others in regard to the prevalence of Types I, II, III and Group IV indicate that such variation occurs. For example Griffith (12) found the incidence of the pneumococcus types in the Smethwick district in England as follows:—

	Type I	Type II	Type III	Group IV
Apr., 1920–Jan., 1922.	30.6	32.6	6.6	30.0
Feb., 1922–Oct., 1924.	42.6	21.3	3.2	32.7
Nov., 1924–Mar., 1927.	34.3	2.4	4.4	53.7

In the third period there was a decrease in Type II cases and an increase in Group IV cases. An interesting study which was carried out with several antisera other than those generally used is that reported by Ordman (13) where different types of pneumococcus as well as different organisms were found predominating in the pneumonias of the Witwatersrand miners in South Africa during a period of 15 years.

An observation that some of the types, found most frequently in the pneumonias of adults, were found seldom in children and vice versa needs study. The theory that children become infected with the types which are most widespread in normal individuals is not supported by the facts observed; for example, although Type VI a, one of the types most frequently found in normal individuals, was one of the

most prevalent in the pneumonias of children, Type I, which was c[...]
paratively rare in normal individuals, also was very frequently fou[...]
The observation that the majority of the types found in normal i[...]
viduals are not found in the pneumonias of adults may be expla[...]
in different ways; as by a lack of virulence or invasive power of [...]
strains; or, that because of the wide distribution of the strains, [...]
majority of individuals become relatively immune to them in e[...]
life.   Probably different explanations will be necessary for the [...]
havior of different types.

A further study of the invasive powers of pneumococci is nee[...]
The epidemic, among children in an institution, of colds, bronc[...]
and pneumonias caused by Type V showed that this type has inva[...]
capacity and should be classed as an infectious organism.   It se[...]
likely that under ordinary conditions, pneumococci are responsi[...]
for a considerable percentage of mild respiratory conditions and t[...]
when the resistance of individuals is especially lowered, pneumoni[...]
more apt to occur.

Where more than one type of pneumococcus is found, their imp[...]
tance in the infection needs to be investigated.

The problem of the preparation of potent therapeutic antisera a[...]
needs further study.   First, there is the selection of suitable stra[...]
and methods of immunization.   It seems to be the general opin[...]
that strains of the highest virulence are most suitable, an opin[...]
which is probably based on observations that degraded strains sti[...]
late the production of antisera which are often deficient in curat[...]
power.   Contrary to this opinion we have results as to the relat[...]
ability of freshly isolated virulent Type III strains having very la[...]
capsules and of less virulent stock Type III cultures having smal[...]
capsules to stimulate antibodies.   Antisera more potent for b[...]
kinds of strains as determined by laboratory tests were produced [...]
inoculation of the less virulent cultures.   If the pneumococcus p[...]
duces a toxin in significant amount in human pneumonia, then stra[...]
of high toxin-producing power and probably the subcutaneous as w[...]
as the intravenous method of inoculation should be used.   Howev[...]
at present, we believe convincing evidence of the existence of su[...]
toxin has not been presented.

SUMMARY

The unclassified strains known as Group IV have been separated into twenty-nine types which are designated by the Roman numerals IV and XXXII. Only a small percentage of the pneumococcus strains isolated in New York City for this study were left unclassified.

The majority of the types gave very slight cross-reactions, the exceptions being Types II and V, III and VIII, VII and XVIII and XV and XXX.

In the series of cases studied, Types IV, V, VII and VIII were found more prevalent in the lobar pneumonia of adults and Types V, VI a and XIV in children.

The majority of the types were also found in normal individuals and in persons having respiratory infections other than pneumonia. Types VI a and XIX were most prevalent in the limited number of strains studied by us.

Fourteen of the types were found in pneumococcus meningitis; Type XVIII was found most often.

Antisera suitable for clinical trial have been prepared for fourteen types. From the majority of the horses inoculated for more than a year, antisera having 500 to 1000 units per cc. were obtained. Antisera of lower potency were concentrated and preparations obtained equal to or stronger than high grade unconcentrated serum.

Potent bivalent antisera have been prepared for types which were found to give marked cross-agglutination reactions.

The results with each type as to prevalence, severity of cases, presence in normal individuals, and in spinal meningitis, potency of antisera produced for therapeutic trial and virulence of strains for mice have been considered under the different type headings.

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QUANTITATIVE STUDIES ON THE PRECIPITIN REACTION

The Determination of Small Amounts of a Specific Polysaccharide*

By MICHAEL HEIDELBERGER, Ph.D., and FORREST E. KENDALL, Ph.D.

(From the Department of Medicine, Presbyterian Hospital, and the College of Physicians and Surgeons, Columbia University, New York)

On the basis of the writers' study of a typical example of the precipitin reaction (1), it has proven possible to employ this reaction for the quantitative estimation of antibody (2–4). The present application of the precipitin reaction to the determination of the other component of the system, namely, the antigen or hapten, arose from the necessity of analyzing solutions containing minute amounts of specific polysaccharides in diffusion experiments designed to throw light on the molecular weights of these substances. The method has been worked out only for the specific polysaccharide of Type III pneumococcus and its homologous antibody, but since it depends upon the standardization of an antibody solution or serum by determining the amount of nitrogen precipitated under definite conditions by known quantities of specific polysaccharide, it should be applicable to any specific carbohydrate obtainable in a state of purity.

It has been shown (1) that the specific polysaccharide of Type III pneumococcus (subsequently referred to as S III) reacts with homologous antibody to give a precipitate consisting largely of protein, and that the precipitate may be considered as a mixture of definite chemical compounds of S III and antibody, the composition of which varies with the relative proportions of the S III and antibody originally present. For this reason, the quantity of antibody protein precipi-

tated is not directly proportional to the amount of S III present. It is therefore necessary to standardize a given antibody solution or serum with known amounts of S III, after which the nitrogen precipitated may be plotted against the amount of S III used. The quantity of S III in an unknown solution is then read from the resulting curve after a determination of the amount of nitrogen it precipitates from the same standardized antibody solution or serum. A precaution to be observed throughout is that the amount of specific polysaccharide used for the determinations be sufficiently small that antibody remain in excess; in other words, that the "equilibrium point" (1), at which S begins to appear in the supernatant, be not exceeded.

Since some of the solutions it was desired to analyze contained high concentrations of salt, all of the determinations, including those necessary for the standardization, were carried out at a concentration of 5 per cent of sodium chloride.[1] This gave the added advantage that the extreme values of the $\dfrac{N}{S}$ ratios in the precipitate were not nearly as far apart as in 0.9 per cent saline. The theoretical aspects of this displacement of the reaction equilibrium by salt will be discussed in another communication.

EXPERIMENTAL

Procedure.—An antibody solution was prepared according to Felton's most recent method (5)[2] from a Type III antipneumococcus horse serum, supplied through the courtesy of Dr. Wm. H. Park and Miss Georgia Cooper of the New York City Department of Health. The solution contained 2.8 mg. of nitrogen per cc., one-half of which was precipitable with S III. 1 cc. was pipetted into an 8 cc. Wassermann tube. 2 cc. of 10 per cent sodium chloride solution and 1 cc. of 0.9 per cent saline containing an appropriate amount of S III were added. The contents were mixed thoroughly by swirling the tube and then incubated for 2 hours at 37°. After standing in the ice box overnight the tubes were whirled in a refrigerating centrifuge at a speed high enough to throw the precipitate down in a compact mass. The supernatant was poured off and the precipitate was

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[1] Actually 5.45 per cent, since an equal volume of 10 per cent salt solution was added to solutions already containing 0.9 per cent salt or its equivalent in phosphate.

[2] Kindly placed at the writers' disposal by Dr. Felton in advance of its publication.

washed with 4 cc. of cold 0.9 per cent saline, swirling the tube to break up the deposit and wash it thoroughly. After centrifuging again in the cold the precipitate was washed again with 2 cc. of saline and centrifuged as before. The precipitate was then suspended in 1 cc. of saline and dissolved by the addition of a few drops of normal sodium hydroxide solution. The solution was washed quantitatively into a micro Kjeldahl flask and the nitrogen determined by a modification of the Pregl micro Kjeldahl method. A blank·was run by making a determination as described without the addition of S III and the small figure

TABLE I

Effect of Repeated Washings on Amount of Precipitate

No. of washings	Nitrogen in precipitate
	mg.
1	0.376
	0.376
2	0.360
	0.364
	0.358
	0.358
3	0.356
	0.356
	0.358
	0.344
4	0.356
	0.356
	0.356
	0.308*

* Precipitate not compact enough after last washing.

obtained was subtracted from the values found. All determinations were run at least in duplicate, and calibrated pipettes were used for the S III and antibody solutions.

The following experiments show that two washings are sufficient to remove occluded protein from the precipitate, and that the amount of nitrogen in combination in the precipitate is not changed by washing with 0.9 per cent saline.

TABLE II

Effect of Washing on Composition of Precipitate

S III added	Nitrogen in supernatant	Nitrogen in original solution	Nitrogen in precipitate by difference	Nitrogen found in washed precipitate
mg.	*mg. per cc. original antibody soln.*	*mg.*	*mg.*	*mg.*
0.075	1.75	2.82	1.07	1.07
	1.75	2.82	1.07	1.07
0.10	1.56	2.82	1.26	1.30
	1.56	2.82	1.26	1.27
0.15	1.38	2.82	1.44	1.44
	1.38	2.82	1.44	1.43

TABLE III

Nitrogen Precipitated by S III from Antibody Solution B 31

S III	Nitrogen found	Nitrogen calculated from empirical equation
mg.	*mg.*	*mg.*
0.01	0.18	0.180
	0.18	
0.02	0.35	0.348
	0.35	
	0.34	
	0.36	
0.04	0.66	0.648
	0.67	
0.05	0.78	0.780
	0.77	
	0.77	
	0.73	
0.075	1.07	1.057
	1.07	
0.10	1.25	1.260
	1.29	
	1.26	
0.15	1.43	1.440
	1.42	
	1.46	

1. Twelve tubes were set up as described above, each containing 1 cc. of antibody solution B 26[3] and 0.025 mg. of S III. The precipitates in two tubes were washed once with 4 cc. of cold saline, and in the other sets of tubes two, three and four times, respectively, using 2 cc. of saline for each washing after the first. The results are shown in Table I.
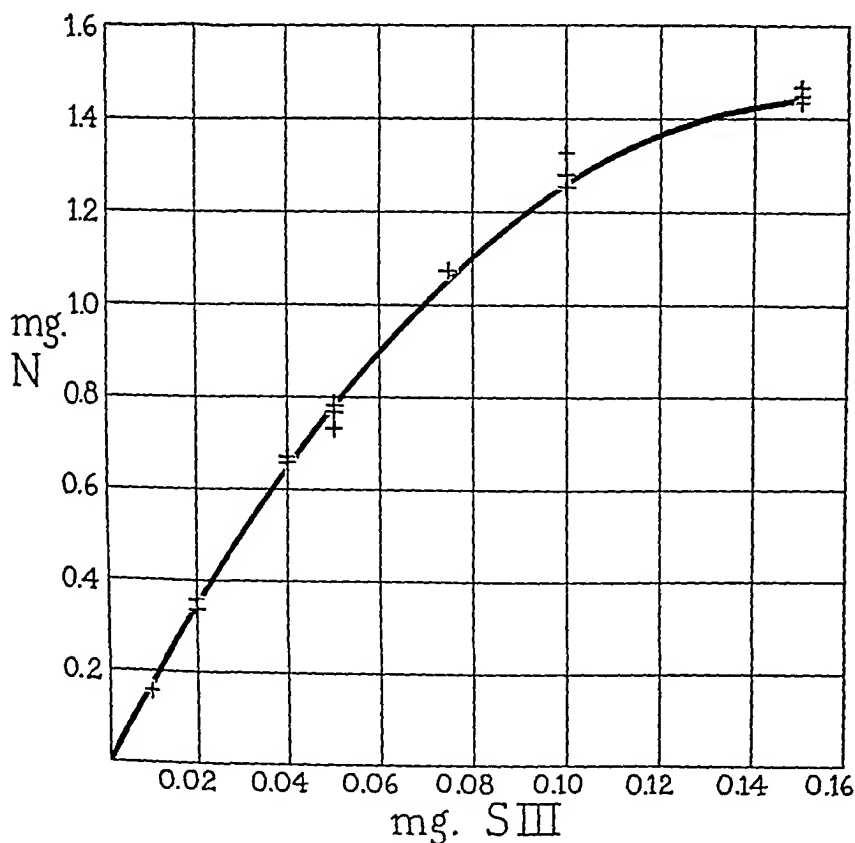
FIG. 1

2. Tubes were set up in duplicate, using 1 cc. of antibody solution B 31 and 0.075, 0.10 and 0.15 mg. each of S III. The nitrogen content of the precipitate was determined both by the method described above and by difference; that is, by subtracting the quantity of nitrogen found in the supernatant from the amount

[3] Made from serum obtained through the courtesy of Dr. A. B. Wadsworth and Miss Mary Kirkbride of the New York State Department of Health Laboratories.

present in the original antibody solution. The agreement between the two sets of values shows that the amount of combined nitrogen in the precipitate is not changed by washing.

Standardization of the Antibody Solution.—Antibody solution B 31 was standardized by determining the amount of nitrogen precipitated by known quantities of S III, using the procedure described above.

TABLE IV

Analysis of Unknown Solutions of S III

Solution	Sample	Nitrogen in precipitate	S III in sample	Total S III	Mean total S III
	cc.	*mg.*	*mg.*	*mg.*	*mg.*
D$_3$, vol. 20 cc.	2.0	1.38	0.125	1.25	
		1.34	0.115	1.15	
	1.0	0.93	0.063	1.26	
		0.93	0.063	1.26	
	0.5	0.51	0.031	1.24	
		0.52	0.031	1.24	
					1.23
D$_4$, vol. 20 cc.	2.0	1.36	0.120	1.20	
		1.34	0.115	1.15	
	1.0	0.89	0.059	1.18	
		0.88	0.058	1.16	
	0.5	0.52	0.031	1.24	
		0.52	0.031	1.24	
					1.20

The results are shown in Table III. The amounts of nitrogen precipitated were plotted as ordinates against the corresponding values of S III as abscissae and a smooth curve was drawn through the points. The equation for the curve was derived and found to be, $N = 18.6 S - 60 S^2$. It is to be emphasized that the equation in this form is applicable only to the antibody solution in question. The derivation and significance of equations of this type will be discussed in the communication already referred to. The calculated curve is given in Fig. 1 and the analytically determined points are indicated

by crosses. The calculated values on the basis of this equation are shown in Table III, together with the values actually found.

The method described above was used to determine the S III content of numerous solutions, each of which contained the amount of S III that diffused through a porous glass plate from a 1 per cent solution in 24 hours. The total volume of each diffusate was 20 cc. In the two examples given, which were from diffusions in 0.05 M phosphate solution, duplicate analyses were made on samples of 2 cc., 1 cc. and 0.5 cc. The nitrogen in the precipitates was determined and the corresponding quantity of S III read from the curve in Fig. 1. The results are shown in Table IV.

SUMMARY AND CONCLUSIONS

A method, based on the precipitin reaction, is given for the micro determination of the specific polysaccharide of Type III pneumococcus. As little as 0.01 mg. of S III can be determined. The method should be applicable to any specific polysaccharide upon standardization of an homologous antibody solution or antiserum in the region of excess antibody.

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STUDIES ON THE CULTIVATION OF THE TYPHUS FEVER RICKETTSIA IN THE PRESENCE OF LIVE TISSUE

By CLARA NIGG, Ph.D., and K. LANDSTEINER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATE 24

This investigation was prompted by the recent significant work of Mooser on the Mexican type of typhus fever. The results on the cultivation of the rickettsia of typhus fever which have already been briefly presented in a preliminary communication (1) are reported in detail in this paper.

Various attempts had previously been made by other workers (Kuczynski (2), Krontowski and Hach (3), Wolbach and Schlesinger (4), Rix (5), Zinsser and Batchelder (6) (*cf.* Zinsser and Castaneda (7))), to cultivate typhus fever rickettsiae. Some of these experiments indicated multiplication of the organisms but none led to the establishment of strains which could be maintained indefinitely *in vitro*. The methods used were practically the same; namely, the cultivation in homologous plasma of tissues (generally brain and spleen) from typhus-infected guinea pigs. In such cultures Kuczynski, Wolbach and Schlesinger, and Zinsser and Batchelder were able to demonstrate rickettsiae morphologically, and Wolbach and Schlesinger succeeded in setting up a second generation, which was done by transferring the same piece of tissue into fresh medium.

Since our preliminary article, Sato (8) reported the cultivation of the virus of typhus fever through thirteen generations, using infected, along with normal Descemet's membrane of rabbits, in a medium consisting of aqueous humor and plasma. The liquid was changed every 2 days and the tissue itself transferred to fresh medium when growth ceased, as it did after from 4 to 10 days. The virulence of such cultures was tested by injecting a suspension of cultivated tissue fragments intracardially. Such injections were followed by fever, monocytosis, and pathological changes consisting of dark red discoloration and edema of the spleen, as well as typhus nodules in the brain, liver, and heart muscle. The author was never able to demonstrate rickettsiae morphologically in his cultures, but described cell inclusion bodies ("*monokokkenförmige Körperchen*") which he identified with the etiological agent in typhus, considering them to be a peculiar form of rickettsia which does not stain with Giemsa or at most very slightly.

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Still more recently Pinkerton and Hass (9) described the cultivation of typhus rickettsiae from the testicle of an infected guinea pig. They used as explant material, small fragments of the membranous exudate imbedded in 1 drop of plasma coagulated by 1 drop of embryonic guinea pig tissue extract. The cultures were transplanted every 2 or 4 days by transferring a portion of the tissue into fresh medium. Rickettsiae were demonstrated morphologically in these cultures in histological sections. They state that in the majority of cases, the rickettsiae, whilst numerous in the first generation cultures, disappeared quite rapidly in successive transfers, but in one group of cultures rickettsiae were found in very great numbers in the fourth and fifth generations after 16 and 21 days *in vitro*.

In the following, a description is given of the technic and the media which have been used in carrying cultures of typhus fever rickettsiae for months *in vitro* without diminution either in virulence or in the number of organisms.

Technic

Typhus Virus.—The strain of typhus organisms used in these studies (unless otherwise indicated) was isolated from a case in the Southeastern United States by the U. S. Public Health Service in Washington, D. C.[1] It is in all respects quite similar to the Mexican strain of Mooser. A few experiments were also made with a strain from Nicolle's laboratory in Tunis.[2] We have carried the latter strain for some months, transferring sometimes with brain emulsions and sometimes with tunica washings. It may be noted parenthetically, in confirmation of Pinkerton's (10) observations on Wolbach's European strain, that in our hands the Nicolle strain produced, although irregularly, scrotal inflammation of slight to moderate intensity, indistinguishable from that produced by the Mexican type. Rickettsiae, although few in number, could also be demonstrated in the testicular exudate when such was present.

Media and Cultures.—Two types of tissue media have been used with equal success. One was adapted from that employed by Rivers, Haagen, and Muckenfuss (11) who used tissue cultures of rabbit cornea in coagulated plasma for the cultivation of the viruses of vaccinia and herpes. For our cultures, pieces (from 2–5 mm. square) of normal tunica from half-grown guinea pigs were soaked in the inoculum suspension, prepared as described below, for 20–30 minutes in order to insure intimate contact between virus and tissue; then, one to three

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[1] We wish to express our gratitude for this material to Dr. G. W. McCoy, Director of the U. S. Public Health Service.

[2] This material was furnished us through the courtesy of Dr. Harry Plotz and Miss Helen Van Sant to whom we are greatly indebted.

pieces were imbedded, according to the directions given by Rivers, Haagen, and Muckenfuss (11), in large tubes (10 cm. long and 2.3 cm. wide) in a small amount of heparinized guinea pig plasma coagulated by means of Ringer solution extracts of normal guinea pig spleen.

The second medium was based on that used by Maitland and Maitland (12), Rivers, Haagen, and Muckenfuss (13), and others for the cultivation of vaccinia virus. This medium consists of Tyrode solution, serum, and minced tissue. For our work, cultures were prepared as follows: Minced normal tunica from half-grown guinea pigs was soaked in a few drops of inoculum for some minutes, after which Tyrode solution and guinea pig serum were added in the ratio of two parts of the former to one of the latter and the mixture distributed in amounts of about 3 cc. into 25 cc. Erlenmeyer flasks.

The tubes and flasks were closed with rubber stoppers and sealed with paraffin to prevent evaporation.

The cultures were incubated at 37.5°C. and transferred at 8–10 day intervals.

Inoculum for the Cultures.—To initiate cultures, the tunica containing rickettsiae from an infected guinea pig was scraped in a few cc. of Ringer or Tyrode solution, or ground in a heavy Pyrex 50 cc. centrifuge tube with a glass rod (inserted through a sterile gauze stopper) terminating in a ball deeply cross-hatched to make an effective grinding surface. The slightly turbid fluid thus obtained was used for inoculation. To transfer the cultures from one generation to the next, part of the tissue was removed from the medium and scraped or ground as above with a few drops of the fluid, and the cloudy suspension used to inoculate fresh tissue for several cultures. Tissue fragments were never transferred, only the supension obtained by scraping.

Stained Preparations.—Preparations for staining were made from the cultures by scraping a bit of tissue on a slide with a cataract knife, then spreading the resulting small amount of turbid liquid into a film which was allowed to dry, fixed in methyl alcohol for 2–3 minutes, again dried and stained with alkaline Giemsa in jars. Good staining was obtained in 15–20 minutes, after which time the slides were washed in running tap water, then rinsed with ethyl alcohol and xylene.

Although the Castaneda stain (14) was found to be excellent for demonstrating rickettsiae in the testicular exudate of infected guinea pigs, where the organisms are found largely within the cellular cytoplasm, it did not give as clear pictures with the scrapings of culture material where there were few or no tissue elements serving as background of contrasting color.

Tests for Virulence.—To test the virulence of the cultures, tissue was removed from the medium and ground with a small amount of liquid. This, along with the tissue debris, was injected intraperitoneally into guinea pigs. Marked scrotal swelling, characteristic temperature curve, and the subsequent demonstration of rickettsiae in the testicular exudate were used as indications of the virulence of the cultures.

Cultivation

It is apparently quite easy to establish cultures of typhus rickettsiae *in vitro* by means of either of the methods described; namely, the coagulated plasma medium or the serum Tyrode medium. All of six strains initiated by the former method and seven of thirteen by the latter were successful. With more careful selection of infectious material from lesions, the failures could doubtless be considerably reduced.

The tissue fragments imbedded in coagulated plasma began to show outgrowths within 2 or 3 days, reaching the maximum in 5–6 days, the final growth being plainly visible to the naked eye as a halo 1–1.5 mm. wide, surrounding the tissue.

Maitland and Maitland (12) originally believed that their serum Tyrode medium does not contain living cells. Rivers, Haagen, and Muckenfuss (13) have shown that the tissue, although it did not proliferate, was none the less viable for at least 5 days, and capable of proliferation when transplanted into a suitable medium. While it is uncertain whether the tissue in the typhus cultures was still viable at the end of 10 days, the incubation period which was commonly used, the rickettsiae certainly were. No systematic experiments have been made to determine precisely how long the organisms can survive without transfer.

Inasmuch as the coagulated plasma method is somewhat more arduous, it was discontinued in favor of the serum Tyrode medium after the latter was found to support growth as satisfactorily as the former. Cultures have been carried in the latter medium through twenty generations covering a period of 6 months, without diminution in numbers or virulence, and there seems to be little doubt that they can be carried indefinitely as any bacteriological culture.

Chart 1 shows the course of a characteristic infection with the cultivated rickettsiae and indicates the subsequent immunity to the passage strain.

Few to fairly numerous rickettsiae could always be demonstrated in stained preparations from the first generations in both types of cultures, their number increasing in later generations, although there was considerable variation in the number of organisms. Fig. 1 shows the characteristic microscopic picture of the rickettsiae in culture.
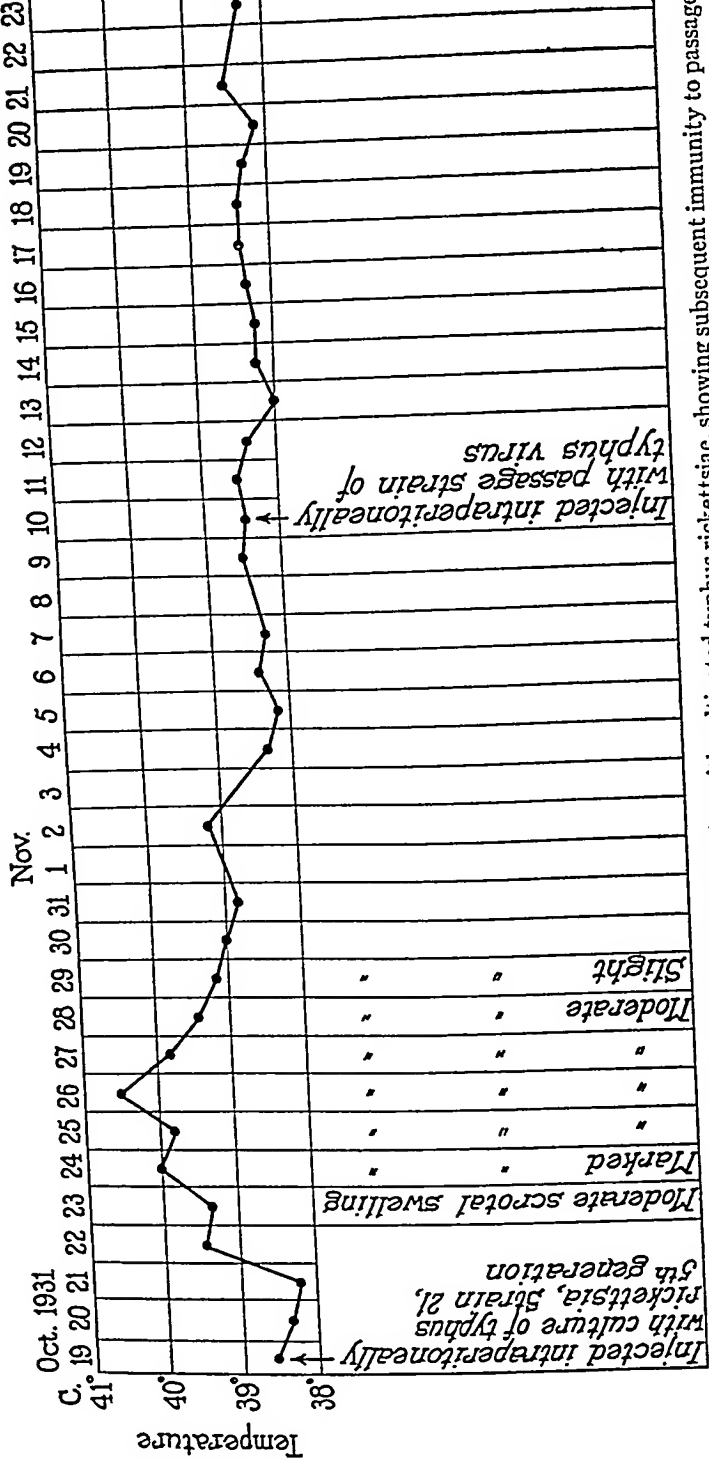
CHART 1. Temperature curve of a characteristic infection with cultivated typhus rickettsiae, showing subsequent immunity to passage typhus virus.

The rickettsiae of European typhus were also successfully cultivated from tunica scrapings of an infected guinea pig in the serum Tyrode medium. In the first generations the rickettsiae in these cultures showed a different morphology (see Figs. 2 and 3), *viz.*, a tendency to form chains of varying length, somewhat resembling minute streptococci, resulting in a picture which raises doubt as to the identity of these organisms. However, apart from the fact that there was no growth in the liquid part of the medium, slides from several of the later generations were in all respects similar to those of the Mexican type, and the strain proved to be fully and characteristically virulent on injection into guinea pigs. No growth was obtained on ordinary media with material from this or the Mexican strain.

The fact that the organisms could be demonstrated morphologically in the serum Tyrode medium only in the scrapings from the tissue fragments, and never in the supernatant liquid, would seem to indicate a parasitism of the rickettsiae for the tissue. Guinea pig tests for the infectiousness of the supernatant liquid were equivocal. In this respect the cultures differ from those of some filterable viruses grown in similar media, in that the latter can be transferred by using the liquid (Maitland and Maitland (12), Li and Rivers (15), Rivers (16)).

Experiments with Anaerobiosis and with Heated and Frozen Tissues

The significance of live tissue is indicated in the results of the following experiments in which heating, freezing, and anaerobiosis were studied as to their influence on the cultures.

The tests for virulence were made with the second generation cultures in the various media, since there was the possibility of a survival of rickettsiae in the inoculum of the first generation.

(a) Minced tunica, suspended in a small amount of Tyrode solution, was heated in a water bath maintained at 50°C. for 15 minutes (*cf.* Pincus and Fischer (17)). This heated tissue was subsequently inoculated and distributed in flasks in the usual manner. Appropriate controls were prepared simultaneously. The tests and controls were transferred after 10 days' incubation, the former again to heated tissue medium, and the latter to unheated tissue medium. Giemsa-stained preparations were made at the time of transfer and again at the end of the second incubation period of 10 days, at which time animals were injected. The results of these experiments are given in Charts 2 *a* and *b*. The numbers in parentheses indicate the generations of the strain.

It will be noted that rickettsiae could not be demonstrated microscopically in any of the cultures prepared with heated tissue, either after the first or the second generation. However, of the three guinea pigs inoculated with material from second generations, one had a typical infection and was subsequently shown to be immune to the passage strain. A second guinea pig showed no reaction but was later found to be immune, indicating an unapparent infection, whilst only one of the three showed no signs of infection nor immunity. These results suggest two possible explanations: firstly, that heating at 50° for 15 minutes is slightly less than lethal for the tissue used; secondly, that the rickettsiae can remain viable without apparent multiplication for 20 days after being transferred into the heated tissue medium.

(b) Minced tunica, suspended in a small amount of Tyrode solution, was frozen with CO₂ snow and alcohol and thawed, fifteen times, after which it was inoculated and distributed in the usual manner. Appropriate controls were prepared simultaneously. Transfers, stained preparations, and guinea pig injections were carried out as in the previous experiments with heated tissue. The microscopic findings and the results of animal tests are shown in Chart 3.

It is seen that tissue killed by repeated freezing and thawing failed to support the growth of the rickettsiae.

(c) Normal tunica inoculated in the usual manner was divided between two series of flasks. One series was stoppered and paraffined in the usual manner to serve as controls. The flasks of the other series were identical except for a longer neck into which a two-hole rubber stopper was fitted, carrying one short and one long glass tube, the latter reaching almost to the surface of the medium. Air was replaced in these flasks by passing hydrogen gas through the long tube, the short tube serving as exit. After the air was driven out (5–10 minutes), the long tube was raised (with the stopper still in place) beyond a constriction previously made in the neck of the flask. With the hydrogen passing through, the flasks were sealed at the constriction in an oxygen flame. Transfers were made after 10 days' incubation into media subjected to anaerobiosis as described above, the controls being prepared as usual. The microscopic findings and results of animal tests are shown in Chart 4.

It will be noted that in these experiments the rickettsiae failed to multiply in the tissue medium under strictly anaerobic conditions. Should this effect prove to be constant, it may be attributable either to a deleterious effect on the tissue or directly on the organisms.

Cultures with Heated Tissue

Strain 21
(3) R. fairly numerous to numerous

(4) R. absent

(5) R. absent

(5) R. absent

(5) R. absent

(5) R. absent

Guinea Pig 64

No infection. Not immune to passage strain

(4) R. absent

(5) R. absent

(5) R. absent

Guinea Pig 63

No apparent infection but immune to passage strain

R. = rickettsiae.

CHART 2 *a*

Control Cultures with Normal Tissue

Strain 21
(3) R. fairly numerous to numerous

(4) R. fairly numerous

(5) R. numerous

(5) R. few to fairly numerous

Guinea Pig 65

Characteristic infection with subsequent immunity

(4) R. fairly numerous

(4) R. numerous to very numerous

Cultures with Heated Tissue

Strain 22
(3) R. numerous

(4) R. absent

(5) R. absent

(5) R. absent

Guinea Pig 79
Characteristic infection with subsequent immunity

Control Cultures with Normal Tissue

Strain 22
(3) R. numerous

(4) R. fairly numerous

(4) R. few

(5) R. fairly numerous

(5) R. few to fairly numerous

Guinea Pig 78
Characteristic infection with subsequent immunity

CHART 2 *b*

CHARTS 2 *a* and 2 *b*. Effect of heating the tissue in the culture medium.

*Control Cultures
with Normal Tissue*

Strain 21
(3) R. fairly numerous
to numerous

(4) R. numerous
to very
numerous

(5) R. fairly
numerous

Guinea Pig 62
Characteristic
infection with
subsequent
immunity

(4) R. fairly
numerous

(5) R. numerous
to very
numerous

(4) R. fairly
numerous

(5) R. numerous
to very
numerous

*Cultures with
Frozen Tissue*

Strain 21
(3) R. fairly numerous
to numerous

(4) R. absent

(5) R. absent

(4) R. absent

(5) R. absent

Guinea Pig 60
No infection
Not immune to
passage strain

(4) R. fairly numerous
to numerous

(4) R. absent

(5) R. absent

(5) R. absent

Guinea Pig 61
No infection
Not immune to
passage strain

CHART 3. Effect of freezing the tissue in the culture medium. Other experiments with frozen tissue gave identical results.

CHART 4. Effect of anaerobiosis on cultures.

Control Cultures
with Normal Tissue

Strain 21
(3) R. fairly numerous
to numerous

(4) R. numerous
to very
numerous

(5) R. fairly
numerous

Guinea Pig 62

Characteristic
infection with
subsequent
immunity

(4) R. fairly
numerous

(5) R. numerous
to very
numerous

(4) R. fairly
numerous

(5) R. numerous
to very
numerous

Cultures with
Frozen Tissue

Strain 21
(3) R. fairly numerous
to numerous

(4) R. absent

(4) R. absent

(5) R. absent

Guinea Pig 60

No infection
Not immune to
passage strain

(4) R. fairly numerous
to numerous

(4) R. absent

(5) R. absent

(5) R. absent

(5) R. absent

Guinea Pig 61

No infection
Not immune to
passage strain

CHART 3. Effect of freezing the tissue in the culture medium. Other experiments with frozen tissue gave identical results.

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Cultures Incubated Anaerobically

Strain 22

(4) R. numerous to very numerous

(5) R. numerous to very numerous

(5) R. absent

(6) R. absent

(6) R. absent

Guinea Pig 88

Temperature 40.2°C. —6th day—no scrotal reaction
Not immune to passage strain

(5) R. absent

(6) R. absent

(6) R. absent

Guinea Pig 86

No infection
Not immune to passage strain

Control Cultures Incubated Aerobically

Strain 22

(4) R. numerous to very numerous

(5) R. numerous

(5) R. few to fairly numerous

(6) R. fairly numerous

(6) R. fairly numerous

Guinea Pig 84

Characteristic infection with subsequent immunity

(5) R. fairly numerous

(6) R. fairly numerous to numerous

(6) R. fairly numerous to numerous

Guinea Pig 85

Characteristic infection with subsequent immunity

CHART 4. Effect of anaerobiosis on cultures.

Some preliminary experiments were made in order to study further the conditions necessary for cultivation.

It was found that of several tissues tested, only tunica and peritoneum gave satisfactory results in the medium described. The peritoneum, because of its greater surface, offers technical advantages in preparing rickettsia cultures on a larger scale.

A few attempts to cultivate the typhus organisms in a cell-free medium similar to that applied by Eagles and McClean (18) for vaccinia virus, were carried out with centrifuged Tyrode extracts of guinea pig kidney. So far these attempts have been unsuccessful inasmuch as no rickettsiae could be found and no infections could be induced in guinea pigs with these cultures.

Although the preceding results seem to indicate that live tissue is the significant constituent of the media described for the cultivation of the typhus rickettsiae, experiments to maintain cultures in the absence of serum have thus far been unsuccessful. In such experiments a medium was employed consisting only of tissue suspended in Tyrode solution, such as Li and Rivers (15) and Rivers (16) found to be entirely adequate for carrying cultures of vaccine virus. Actually rickettsiae were found in first generations in such a medium but usually reduced in number—and were for the most part absent in the second generations. In one such experiment rickettsiae were demonstrable in two generations, but not in the third. Although serum could not be entirely eliminated from the medium, it was found that the quantity could be reduced to at least half of that used as routine (*i.e.* one part of serum to five parts of Tyrode solution, instead of one to two parts) without damaging the cultures. It seemed to make little difference whether the serum was diluted with Tyrode, Ringer, or ordinary physiological saline. Whether the function of the serum consists merely in prolonging the viability of the tissue, has not been determined.

COMMENT

Although the etiological rôle of *Rickettsia prowazeki* in typhus fever hardly needs further confirmation, it is substantiated by the fact that guinea pigs recovered from infections, entirely typical of experimental typhus, produced by the injection of cultures as herein described, are

immune to passage virus. Moreover, rabbits infected with such cultures developed positive Weil-Felix sera.

A significant outcome of the experiments is to be found in the similarity of the growth conditions of *Rickettsia prowazeki* and filterable viruses. In general, the presence of living tissue is considered to be necessary for the cultivation of viruses. This is stressed by Rivers (19) (*cf.* Dale (20)), and was recently substantiated in a paper by Hallauer (21) on the cultivation of the virus of fowl plague (*cf.* Landsteiner and Berliner (22)). This relation of viruses to live tissues has been used as one of the arguments in favor of the view that viruses are not living organisms. It is of interest, therefore, that similar conditions for growth obtain in the cultivation of rickettsiae which, on account of their morphology, certainly must be deemed living microbes.

SUMMARY

1. *Rickettsia prowazeki* can be cultivated for many generations *in vitro*, without diminution in numbers or virulence, in media similar to those described by Maitland, Rivers, and others for the cultivation of certain viruses. In all probability, such cultures can be maintained indefinitely.

2. It has been impossible, thus far, to cultivate the typhus rickettsia without employing living tissue.

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EXPLANATION OF PLATE 24

FIG. 1. Typhus rickettsiae in culture, sixth generation. Giemsa stain. × 1000.

FIG. 2. From a culture of European typhus, fourth generation, showing chains intra- and extracellularly. Giemsa stain. × 1500.

FIG. 3. From a culture of European typhus, sixth generation, showing long chains intracellularly. Giemsa stain. × 1000.

(Nigg and Landsteiner. Cultivation of typhus fever rickettsia.)

THE AGE FACTOR IN THE VELOCITY OF THE GROWTH OF FIBROBLASTS IN THE HEALING WOUND*

By EDWARD L. HOWES,† M.D., AND SAMUEL C. HARVEY, M.D.

(From the Department of Surgery, Yale University School of Medicine, New Haven)

Wounds heal with greater rapidity in the young than in adults. Du Noüy has (1) demonstrated this experimentally. He found that in epithelial wounds of equal size the rate of healing varied in inverse ratio to age; *i.e.*, the more rapid rate was in the young and aging constantly diminished the velocity. An increased rate of cellular proliferation has generally been accepted as the explanation for the greater rapidity of healing, particularly as growth is characteristic of this period of life. It was suggested, however, in a previous paper by one of us that this increased rate of healing might be attributable to another cause,—a diminution in the amount of retardation entering into the process. This retarding factor is apparently what limits the mass of tissue regenerated to a volume just sufficient to replace the damaged tissue (3), and evidently it plays a large rôle in the healing for the accelerated phase with its increasing daily increments of strength is very brief, as contrasted to the longer retarded phase, with its daily increments of strength continually decreasing.

To determine whether the rate of fibroplasia is actually greater in the wounds of the young and what the relation is between the accelerated and retarded phases of repair is the object of this investigation. A velocity curve of healing for wounds in the stomach of young rats will be derived by the method previously employed for the adult rats and the two curves will then be compared. A comparison will also be made with another velocity curve of healing in which there was a definitely increased rate of fibroplasia (3). This curve was obtained by feeding animals on a high protein diet. Clark was the first investi-

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gator to demonstrate that food could influence the rate of healing (2). He found a shortening of the latent period before healing began in wounds of the skin when the dogs were fed a high protein diet, and the entire length of healing time was correspondingly diminished. A high fat diet had the opposite effect—prolongation of the latent period and an extended healing time.

. EXPERIMENTATION

Healthy young rats of our own stock, weighing from 58 to 85 gm. and varying in age from 35 to 48 days, were used. They were kept in separate cages and fed on the standard diet previously employed in the adult rats (Table I). Until the time of operation they had had normal growth curves.

TABLE I

Standard Balanced Diet (Smith and Moise)

Composition		Calories per kilo of food	Apportionment of total calories	
	per cent			*per cent*
Casein......................	18	738	Protein....................	13.8
Starch......................	51	2,091	Carbohydrate..............	39.2
Crisco......................	22	2,046	Fat.......................	47.0
Cod liver oil.................	5	465		
Salts (Osborne and Mendel mixture)......................	4	.		
Lettuce twice a wk. 70 mg. of yeast daily		5,340		

After being fasted for 6 hours, an incision of approximately 1 cm. in length was made in the cardiac portion of the stomach and sutured in two layers with No. 000 plain catgut. Under these circumstances this size catgut loses its tensile strength in 48 hours (5). The same technique of suturing was always employed, the mucosa being first approximated and then the serosa with slight inversion. The abdominal wall was closed with silk. After this procedure the rats were immediately returned to their cages and the diet. At definite intervals after the operation, five rats were killed and the strength of the wounds determined. Animals were discarded with infected wounds or with pathology in other organs. To test the strength of the wound, the stomach was excised, the esophagus tied off, and a cannula inserted in the pylorus. Air was then admitted at a constant rate of speed from a constant head of pressure. The force required to rupture the wound was registered on a rotating drum by means of a mercury manometer

equipped with a writing point. The details of this technique have been described previously. To test the strength of the holding power of the sutures alone, wounds were repaired and tested in five dead animals.

TABLE II

P. O.	Young rats (standard diet)			Adult rats (standard diet)			Adult rats (protein diet)		
	Average strength	Standard deviation of mean	Nature of break	Average strength	Standard deviation of mean	Nature of break	Average strength	Standard deviation of mean	Nature of break
days									
1	60	±10.5	In incision	41.5	±9.0	In incision	45.4	±4.0	In incision
2	58	±10.5	" "	47.5	±9.0	" "	33.6	±4.0	" "
3	70	±9.0	" "	49.9	±15.0	" "	42.0	±3.0	" "
4	76	±2.0	" "	45.8	±8.0	" "	38.6	±7.0	" "
5	102	±6.0	" "	71.0	±5.0	" "	94.0	±5.4	" "
6	140	±9.0	" "	107.0	±6.0	" "	135.0	±5.0	" "
7	178	±10.0	" "	135.0	±5.4	" "	150.0	±5.3	" "
8	192	±7.0	" "	143.0	±6.8	" "	154.0	±9.0	2 elsewhere 1 alongside 4 incision
9	187	±19.0	2 elsewhere 2 alongside 1 incision	153.5	±10.0	" "	162.0	±8.0	1 elsewhere 2 alongside 3 incision
10	195	±6.0	4 elsewhere 1 alongside	150.5	±7.0	1 alongside	162.0	±4.0	2 elsewhere 3 alongside 0 incision
11	194	±10.0	All elsewhere	166.0	±13.0	2 alongside 3 incision 4 elsewhere	202.0	±4.4	5 elsewhere 1 alongside 0 incision
12				185.0	±15.0	4 alongside 4 elsewhere			
13				183.9	±7.0	None in incision, 7 elsewhere			
14				206.0	±10.0	All elsewhere			

RESULTS

In the gross, the wounds were well healed and relatively free from peritoneal adhesions. Only one infection occurred in the 65 rats employed. Six animals were discarded because of pulmonary abscesses.
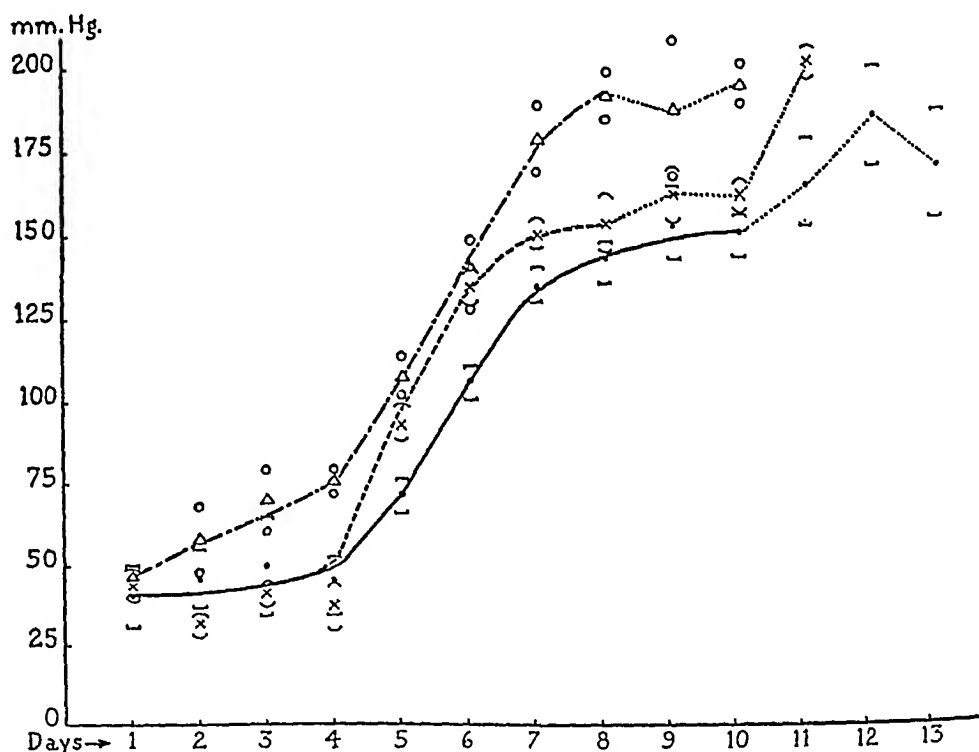
x Mean of observations of adult rat on high protein diet.   ----------- curve.
• Mean of observations of adult rat on standard diet.   ————— curve.
△ Mean of observations of young rat on standard diet.   — · — · — curve.
( ) Standard deviation of mean of adult rat on high protein diet.
[ ] Standard deviation of mean of adult rat on standard diet.
o o Standard deviation of mean of young rat on standard diet.

FIG. 1. Curve of tensile strength of wounds in adult and young rats on standard diet and adult rats on high protein diet.

The average strength of the holding power of the sutures in the dead animals was found to be 25 mm. of mercury. The average strength of the wounds in the animals killed 6 hours after operation was 77 mm. of mercury—a difference of 57 mm. of mercury.

The curve plotted from the data (Table II) with the average strength of the wounds in millimeters of mercury as ordinates and days as abscissae (Fig. 1) had the following characteristics: a latent period with the strength remaining practically at a level of 60 mm. for the first 3 days, followed by an increase in strength until the 8th day. During these 8 days all of the ruptures occurred within the suture line, but on the 9th day, both in the suture line and alongside the incision, and on the 10th day, not in the incision but elsewhere in the stomach. This last day was taken as the end-point.

On studying the intake of these rats it was found that they had consumed 11.5 gm. of the diet per 100 gm. of body weight (Table III).

TABLE III

Calculations Based on the Percentage of Body Weight at Start of Experiment

	Young rats (standard diet)		Adult rats (standard diet)		Adult rats (high protein diet)	
	Start to opera-tion	Opera-tion to sacri-fice	Start to opera-tion	Opera-tion to sacri-fice	Start to opera-tion	Opera-tion to sacri-fice
Difference in weight per day, per cent......	+4.1	+3.1	+0.26	−0.45	−0.3	−0.5
Food consumed per day, per cent.........	12.0	11.0	4.3	2.5	3.3	2.4
Protein consumed per day, per cent........	2.2	2.0	0.75	0.45	2.6	2.0
Calories of food consumed per day, per cent.	59.0	55.0	23.0	13.0	14.0	10.0
Calories of protein consumed per day, per cent.................................	8.1	7.5	3.1	1.7	9.5	6.8

The daily average consumption of food after operation was approximately equivalent to the average consumption before operation, although on the 4th and 6th days afterwards the average was slightly less than on other days. The rats gained 4.1 per cent of their body weight daily before operation and continued to gain at the rate of 3.3 per cent per day after operation.

Comparison of the Velocity of Healing in the Young and the Adult Rat

The average strength of the holding power of the sutures in the wounds of both the young and adult rats was approximately equal. (25 mm.). The suture techniques were therefore comparable. In the young rats killed 6 hours after the operation, the wounds

averaged 77 mm. of mercury as compared to 45 mm. in the older group. This increase in the strength after 6 hours, more manifest in the young, could only be attributed to an alteration in the wound occurring almost immediately after suturing. Anatomically, an increased deposition of fibrin was the only demonstrable change. A difference in the amount deposited might account for the greater strength found in the younger animals. The drop in strength from 77 to 50 mm. after the first 24 hours is an expression of the combined loss of strength of the suture material and diminution in the holding
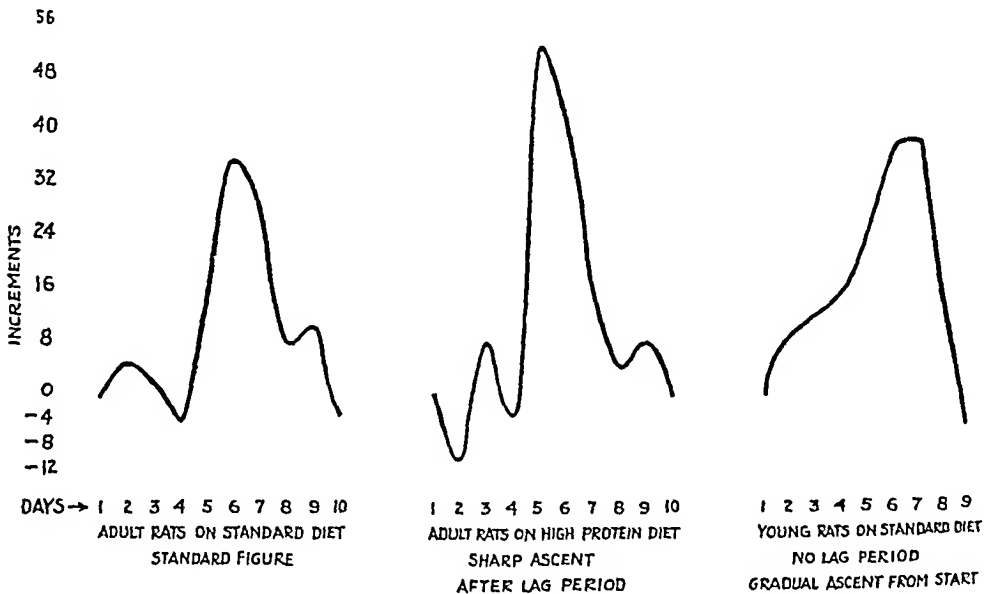
FIG. 2. Curve of daily increments.

power of the tissues. Throughout the latent period an average of approximately 60 mm. was maintained (Fig. 1), and was about 15 mm. higher than that found throughout the same phase in the curve for the adults. Again, as fibrin was demonstrable throughout this latent period and is the scaffold on which the fibrous tissue grows, the greater strength might be attributed to a greater deposition of it. The plus and minus increments of the latent phase, found in all curves, can be explained by the multiplicity of factors influencing the strength of this period.

Once fibroplasia began, however, there were definite increasing positive increments in both curves. This trend began 1 day earlier

in the curve for the young animals, on the 3rd day in contrast to the 4th day in the curve for the adults. The difference was also demonstrable by connective tissue stains. In the wounds of the young rats fibroplastic proliferation was noticeable on the 3rd day while in the wounds of the adults it had not begun on the 3rd day. In spite of this earlier beginning of fibroplasia, its rate in the wounds of the young was no greater than in those of the adults, for the curves were parallel through the 4th, 5th and 6th days. Of course, the 1 day earlier beginning of fibroplasia would account for some shortening of the endpoint of healing in the curve for the young animals, but it would not account for the shortening of 3 days which actually occurred, especially when there were equal rates of fibroplasia in the first portion of both curves. The explanation for this is furnished by the length of the accelerated phase. This period lasted from the 3rd through the 7th day in the young rats or 2 days longer than in the adults where it remained from the 4th through the 6th day. Stated in another way, the retarded phase of healing began 1 day later in the curve for the young animals.

Before the end-point was reached, there was in both curves an irregular portion in which both the strength of the stomach and that of the wound was being tested. This period was 1 day shorter in the curve for the younger rats. During this period in the curve for the adults, the strength staggered upwards to reach a strength equivalent to the average strength of the stomachs. In the curve for the young rats by contrast, this equivalent strength was reached before the irregular period began.

Comparison of the Curve for Young Rats with the Curve for Adult Rats on a High Protein Diet

This latter curve was obtained by the same experimental method, except that the rats were fed on a diet containing 68 per cent protein.

The latent period of the curve for the young rats was 1 day shorter than that for the adults on the high protein diet. Once fibroplasia began, however, the daily increments in the curve for the young rats were much smaller than those for the high protein curve, indicating a diminished rate. The entire phase lasted 4 days in the curve for the young as contrasted to 1 in the curve for the adults. In spite of the

larger increment in this phase on the high protein diet, retardation s
in 3 days sooner than in the curve for the normal adults.   The peric
of approaching maximum strength began on the 9th day and was
only 1 day's duration in the young in contrast to the 8th day and
days' duration in the curve representing the high protein diet.   Tl
end-point of the young curve occurred on the 10th day, while for tl
high protein it fell on the 11th day.

<div align="center">DISCUSSION</div>

The shortening of the period before strength is manifested in woun(
of young rats may be compared to similar findings in other fields (
biology.   Penfold, for example, found a shorter period before tl
multiplication of bacteria began in the transplants from young parer
cultures (6).   Robertson (7) noted an equivalent shortening for ii
fusoria taken from young parent cultures.   With tissue culture
Carrel and Ebeling (8), and Burrows (9) obtained quicker prolifera
tion when they employed embryonic serum rather than serum froi
adults.   It seems, then, that young forms in general have a shorte
period before reproduction or regeneration begins than adults.   Thi
general rule was followed in the wounds of the young animals.

That the rate of fibroplasia is no greater in the wounds of the youn
than in the wounds of adults seems also to find a counterpart in ger
eral biology, where the rates of reproduction of transplants from youn
cultures, such as bacteria and infusoria, are no greater than in th
adult.   Robertson (7), commenting on this said, "If, however, th
lag-periods [that is, the periods before multiplication begins] b
deducted from each, the reproductive rate of individuals isolate(
from an old parent-culture is not less, but may actually be greate
than the reproductive rate of individuals isolated from a young parent
culture."

Without an increased rate of fibroplasia, therefore, shortening o
healing time in the wounds of the young must depend on a diminutior
in the amount of retardation entering into the process.   Let us con
sider how much retardation there is in the curve of healing for th(
young rats as contrasted to the curves for the adult rats.   The onsel
of retardation in each curve differed (Fig. 2).   The phase began 1
day later in the young than in the adults on a standard diet, and 2

days later than in the adults on the high protein diet. The daily amount of retardation calculated by making an algebraic subtraction of each succeeding increment from the one before it shows that the total amount of retardation before the final phase of healing is reached was least in the young (Table IV). For the adult on the standard diet it was 39, for the adult on the high protein 41, and for the young 25. The amount of retardation in the young curve would be even less if it were possible to add the amounts included in the final phase,

TABLE IV

Daily Increments of Strength

Between days	Young curve		Standard curve		High protein curve	
1–2	−2	Latent	+6	Latent	−12	Latent
2–3	+12	period	+2	period	+9	period
3–4	+6		−4		−4	
4–5	+26	Accelerated	+26	Accelerated	+56	Accelerated
5–6	+38	phase	+36	phase		phase
6–7	+38	Retarded	+28		+41	Retarded
7–8	+13	phase	+8	Retarded	+15	phase
8–9			+10	phase		
9–10			−3			
Sum of increments in accelerated phase.....	70		62		56	
Total amount of retardation. (Calculated by subtracting each succeeding increment from one before and adding differences)...........	25		41		39	

for the entire final period is shorter in the curve for the young rats. Here is direct mathematical proof, then, that retardation begins later and there is less of it in the healing of wounds in the young.

Next we must consider what is the explanation of the late appearance of retardation in the curve for the young rats. Is it because of a greater rate of fibroplasia during the accelerating phase of healing? Apparently, an increased rate of fibroplasia during the phase of acceleration does not have the capacity to delay the onset of retardation. In the curve for the high protein diet, for example, there were greater

increments during the phase of acceleration, indicating a greater
of fibroplasia than in any of the other two curves, yet in spite of t
retardation began earlier than in either of the other two. Robertso
theory (7) for retardation in tissue cultures would explain why
early retardation accompanies an increased rate of proliferation. F
has presented evidence to show that regeneration and growth a
autocatalyzed phenomena. At each cell division, he believes ther
is a catalyzer given off by the nucleus of the cell, part of which i
secreted into the pericellular fluid and part of which remains within
the nucleus. As the pericellular fluid becomes saturated with this
catalyzer, the amount given off to the surrounding fluid at each cell
division becomes less, and retardation occurs as its concentration
increases. The end-point is reached when the pericellular fluid be-
comes saturated. The faster this catalyzer accumulates as a result
of rapid proliferation, the sooner retardation occurs. This theory
would explain why there is early retardation in the high protein curve
and why there is late retardation in the curve for the young. It would
also strengthen the viewpoint that there is a less rapid rate of prolifera-
tion in the healing of wounds of the young.

Carrel's and Ebeling's experiments (8) showed that age played a
rôle in retarding the proliferation of cells. Their tissue cultures in
embryonic serum came to a greater mass in a shorter length of time
than in the serum of adults. When they diluted the embryonic serum
to twice its volume, the rate of proliferation was not decreased. A
diminution should have followed if the embryonic serum contained
an accelerating substance. They therefore concluded that there
must be an inhibiting substance in the serum of the adults. The
presence of an inhibiting substance in the blood of the adult rat would
explain why there was an early appearance of the retarded phase in
the healing of their wounds.

The combination of retardation arising from the age factor and from
autocatalysis would adequately explain all the variations in retarda-
tion found in the three curves. In the adults on the normal diet,
retardation from age added to autocatalytic retardation would act
to slow the process earlier than in the young where there was no re-
tardation from age. In the adults under the stimulation of the high
protein diet, the greater autocatalytic retardation plus the retardation
from age would slow the process even earlier than in the adults on the

standard diet and the total amount of retardation would be greater. In the young, all retardation attributable to age could largely be deducted and there would remain only autocatalytic retardation. Accordingly, it should appear later and the total amount should be less. This is actually what is found in these experiments.

Of course, the completion of the healing of the wound is a balance of both the duration and quantity of the accelerated and the retarded phases, and the relation of these phases in the curve for the adult rats on the standard diet must be taken as the norm for the comparison of the phases in the other two curves. A review of the balance of the two phases in comparison to the standard curve will show why the healing times varied. Complete healing was obtained more rapidly on the high protein diet in spite of more retardation because the accelerated phase was quantitatively greater during its existence. The end-point of healing was reached more rapidly in the curve for the young rats not because of greater acceleration either in duration or quantity but because retardation appeared later and was less in quantity.

Naturally, it can be argued that in the curve for the young rats there was an increased rate of fibroplasia—that the increments during the first part of the curve are only equal to those of the adult because retardation has already reduced them to a seeming similarity. However, this argument does not hold for if it were applicable to the young curve it would be equally so for all the curves. We must reason, therefore, from the time at which the increments definitely begin to change.

In all three experiments an effort was made to produce a wound of approximately the same size and to suture it in the same way. Theoretically then, the same amount of fibrous tissue was regenerated and the algebraic sum of the combined forces of acceleration and retardation working antagonistically to complete healing should be approximately the same in all three experiments. How near this was approached can be seen in the following tabulation:

Total accelerating increments.........	Young 70	Standard 62	High protein 56
Total retarding increments...........	" 25	" 39	" " 41
Addition....................	95	101	97

The discussion of retardation in the curves of the healing of the wound inspires the question as to what there is that limits scar tissue in the process of regeneration.   There is little evidence as to what this may be, but there is much evidence as to what it is not.   The work of Carrell (8) suggests that it is referable to the blood plasma.   If so, it is not the exhaustion of food supply, for surely it remained the same in all three experiments.   It is apparently not an accumulation of by-products, for this idea has been refuted many times as an explanation of why bacteria in media when they reach a definite number stop growing.   Hixson (10) found that his yeast cultures repeated their growth cycles if the alcohol were drawn off.   It if is local or more intimately related to the cell itself, it is not the exhaustion of the capacity of the cell to reproduce itself.   Carrel's (11) tissue cultures of heart muscle under favorable conditions for growth have proliferated for years.   Moreover, Osborne and Mendel (12) were able to delay growth by restricting food until the animal was quite old, but then growth was renewed with equal vigor when food was again given.   Here the capacity for regeneration was not lost, even though the supply of food was insufficient.   We know, too, that the cicatrix has the capacity to regenerate again and again, for it will do so in response to many new injuries.   Therefore, the ability of the cells to regenerate is not lost, in the arrest of fibroplasia.   If the retardation is physical, *i.e.* compression on the cells after a certain mass is produced, then it must be remembered that spacial relations are no barrier to keloids, to simple hypertrophies or to malignancies.   These last two conditions are, of course, abnormal manifestations of proliferation of tissues, but they must be considered here for tumefaction must be caused either by an absence of the retardation or an increased rate of proliferation.

The caloric intake of the young rats was practically four times that of the adult (Table III).   Consequently, the amount of protein taken by them as calculated in relation to the body weight was high, in fact equal to that of the adults on the high protein diet.   Yet, this relatively high protein and caloric intake did not increase the rate of fibroplasia as it did in the adults on the high protein diet.   That this high protein, high caloric intake was utilized by the young for growth, daily maintenance and wound healing, while in the adults it was used simply for daily maintenance and wound healing, seems to furnish a

logical explanation of why there was not an increased rate of fibroplasia in the young, especially as even the young controls ate four times as much as did the adult controls. The young rats continued to gain in weight after operation at a rate only slightly less than before. Under similar conditions, the adult rats lost a slight amount.

There is then good evidence to suggest that the shortening of the healing time in young animals is not because of an increased rate of fibroplasia but rather from a diminution of factors retarding the process. Of course, in the final analysis of the entire process of the healing of wounds of the same size in both the young and old, the total amount of retardation must be the same in order to stop both processes. However, in the young animals for the phase of healing tested by this method, a diminished amount of retardation and its late appearance combined with an earlier appearance of fibroplasia allow earlier reacquisition of strength and nothing can be said about the amount of retardation entering into the final organization of the cicatrix.

SUMMARY

The velocity curve of fibroplasia in the healing of wounds in young rats reached its end-point 3 days ahead of a similar curve for adults. Strength and fibroplasia were manifest 1 day sooner than in the adults. A study of the increments of the curve showed that the rate of fibroplasia during the accelerated phase was less in the young and that it lasted longer. Correspondingly, retardation appeared later and was less in amount than in the curve for the adult rats. The amount of retardation was even less than in the curve obtained for adults on a high protein diet, in spite of the fact that in this latter curve there was a definite increase in the rate of fibroplasia.

Healing in the young, therefore, is more rapid than in adults because fibroplasia begins earlier and is less retarded, not because the rate of fibroplasia is greater.

Growth of the young is not hindered by the process of wound healing.

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REACTION OF RABBITS TO STREPTOCOCCI: COMPARATIVE SENSITIZING EFFECT OF INTRACUTANEOUS AND INTRAVENOUS INOCULA IN MINUTE DOSES

By M. P. SCHULTZ, M.D., AND HOMER F. SWIFT, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

It has been demonstrated that chronic low-grade infection, or numerous localized foci of inflammation induced in the rabbit by repeated minute inoculations with relatively avirulent (green or indifferent) streptococci, evoke a state of hypersensitivity to the bacteria (1, 2). This hypersensitivity may be demonstrated by hyperreaction to minute doses injected intracutaneously, by an ophthalmic reaction following application of the bacteria to the scarified cornea, and by death in from 24 to 48 hours after intravenous doses tolerated by normal animals (2). Suitable preliminary intravenous inoculations result in another type of response; the characteristic hyperergic reactions are not induced; skin lesions are smaller than in normal animals and quickly assume a hard nodular character without much macroscopic evidence of edema; ophthalmic tests are negative; the responses are those of immune animals (3).

It is possible that these differences in response of animals to the two modes of preliminary inoculation might be conditioned by a small antibody content in the animals inoculated intracutaneously compared with a larger amount in those inoculated intravenously. The induction of high degrees of bacterial hyperergy with repeated small inocula of streptococci suggested comparing the effect of similar small doses given intravenously; for from both routes a low antibody production might be expected because of the small amount of antigen introduced. Thus, the question as to whether these differences in the reactive state of the tissues might be the result of suitable doses of antigen, rather than of its site of action, focal or general, seemed open to further

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investigation; and the following experiments were designed to throw additional light on this subject.

Method

For the first experiment a vaccine was made from a hemolytic streptococcus, Strain S 43, originally isolated from the throat of a patient with measles. This organism killed rabbits of 2,500 gm. within 24 hours following intravenous inoculation with 1.5 cc. of broth culture. It was preserved in the frozen and dried condition (4) from which fresh blood broth stocks were prepared monthly. Fresh vaccines were made each week from the centrifugate of 20 hour tryptic broth culture, resuspended in Ringer's solution, and heated at 56° for 35 minutes. The purity of the culture and sterility of the vaccines were suitably controlled. In the second experiment, living cultures of *Streptococcus viridans*, Strain V 110 A, originally isolated from a rheumatic subcutaneous nodule, were used. Cultures were preserved and grown in the manner described for Strain S 43; and fresh cultures were used each day, with microscopic and cultural control of purity.

Dilutions for intracutaneous inoculations were prepared in Ringer's solution, in the first experiment from suspensions of heat-killed centrifugate made to the same volume as the original culture, and in the second experiment directly from the cultures. The volume injected was 0.1 cc. in all instances: an inoculation of 10^{-1} cc., therefore, consisted in 0.1 cc. of original vaccine or culture, 10^{-2} cc. of 0.1 cc. of a 1:10 dilution, and 10^{-3} cc. of 0.1 cc. of a 1:100 dilution. The size of lesions produced is recorded in terms of cubic millimeter volume, estimated from measurements of two diameters and the height of each lesion, as described elsewhere (2).

For the ophthalmic reaction (2) cultures were concentrated 50:1 in Ringer's solution, and 0.3 cc. of suspension was instilled in the conjunctival sack after corneal scarification. In the first experiment the suspension was heat-killed; in the second, living cultures were used.

The serum agglutinin titers were determined in the following way: a large volume of tryptic digest broth (1,500 cc.) was inoculated with 50 cc. of an 18 hour culture and grown at 37°C. for 7 hours. After centrifugation the sediment was concentrated (100:1 or more) in plain broth to make a suitable suspension. Reactions were read after 2 hours' incubation at 56°C. These organisms yielded unstable suspensions unless such precautions were taken.

EXPERIMENTAL

Experiment 1.—As indicated in Chart 1, quantities of 10^{-2} cc. and 10^{-3} cc. hemolytic streptococcus vaccines were given daily for 41 days. These doses were injected intracutaneously at two sites in each of the six rabbits of Group A, and a similar amount was given intravenously to the five rabbits of Group B.

At intervals of 1 week the lesions in the skins of Group A were measured. As the chart shows, a fair degree of sensitivity was demonstrable in this group by the 22nd day; but it diminished later and rose again towards the end of the experiment. Such fluctuations are not unusual and occurred also in the second experiment.

On the 45th day the comparative sensitivity of the two groups was tested by intracutaneous inoculations with 10^{-4} cc., 10^{-5} cc., and 10^{-6} cc. of vaccine. These small doses were employed in order to minimize the possible sensitizing

TABLE 1

Experiment 1

Comparison of Groups on 45th Day

Rabbit No.	Cutaneous reaction		Ophthalmic reaction*	Agglutinations						
				1:10	1:20	1:40	1:80	1:160	1:320	1:610
25-69	Group A Intracutaneously sensitized	±	+	±	±	±	−	−	−	−
25-70		±?	±	±	±	−	−	−	−	−
25-71		±?	+	±	±	−	−	−	−	−
25-72		+	+±	+±	+±	±	−	−	−	−
25-73		±	+±	±	±	−	−	−	−	−
25-74		+++	++++	±	±	±	−	−	−	−
25-81	Group B Intravenously immunized	−	+	±	±	−	−	−	−	−
25-82		−	±	+	±	−	−	−	−	−
25-84		−	−	+±	+±	±	−	−	−	−
25-85		−	−	±	−	++	++	±	±	−
25-86		−	−	+±	+±	±	±	−	−	−

Negative reaction indicated: −.
Increasing degree of reaction indicated: ± to ++++.
* Right eyes tested at this time.

effect of intracutaneous inocula in Group B in later periods of the experiment. The results, summarized in Table I, showed Group A to be much more sensitive, both in cutaneous and ophthalmic reactions. The agglutinin content of the sera of the animals in both groups was quite comparable, with possibly slightly more in Group B.

From the 51st to the 69th days, larger doses, usually of 10^{-1} cc. and 10^{-2} cc. were given daily. On the 70th day comparative titrations were again performed, but with inocula ranging from 10^{-1} cc. to 10^{-5} cc. At this time a group of four rabbits previously untreated (Group C) was introduced into the experiment. The results are indicated in Chart 1 and Table II.

Five animals of Group A responded with very large cutaneous reactions,
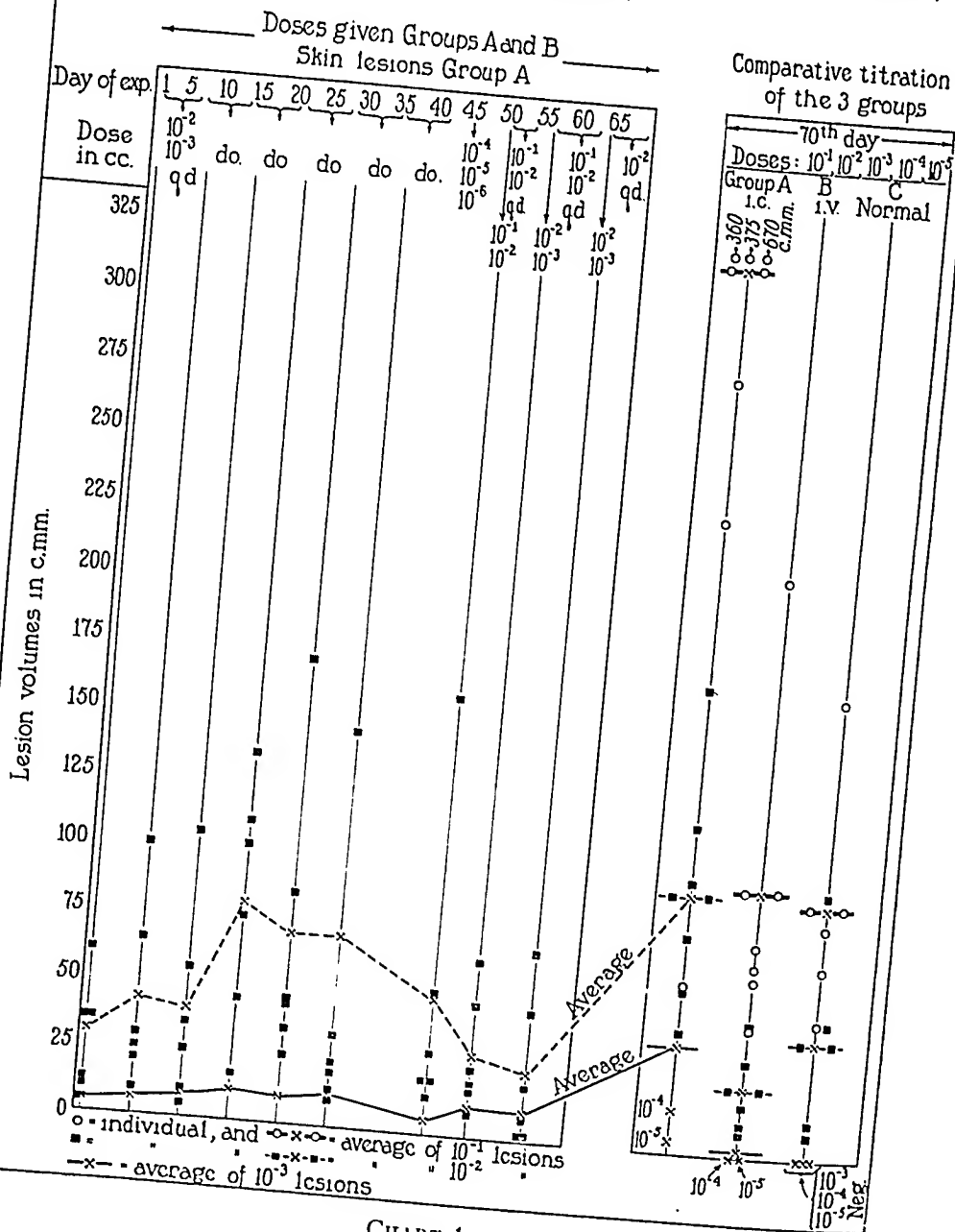
CHART 1

those of Group B with distinctly smaller reactions, but these, on the other hand, averaged very slightly larger than in Group C, normals, inoculated the first time with comparable doses of vaccine. The lesions in Group B were, however, hard and nodular and had very little edema about them at a time when the lesions in Group A were very red and edematous. The ophthalmic reactions indicated that the corneae of the intravenously immunized animals were less sensitive to the irritating action of the vaccine than were those of Group A. The relatively

TABLE II

Experiment 1

Comparison of Groups on 70th Day

Rabbit No.	Cutaneous reaction	Ophthalmic reaction*	Agglutinations							
			1:10	1:20	1:40	1:80	1:160	1:320	1:640	Control
25-69	++++	++	+	±	±	±	—	—	—	—
25-70	+	±	+±	+	±	±	—	—	—	—
25-71	++++±	±	+±	±	±	±	±	—	—	—
25-72	+++	±	+±	+	±	±	—	—	—	—
25-73	++±	±	++	±	—	—	—	—	—	—
25-74	++++	++++	+±	+	—	—	—	—	—	—
			±	±	±	±	±	—	—	
25-81	±	±	+	±	±	±	—	—	—	—
25-82	++	—	+	±	±	—	—	—	—	—
25-84	±	—	++	++	++	++	++	+±	—	—
25-85	±	—	+	±	—	—	—	—	+	—
25-86	±	—	+±	±	±	—	—	—	—	—
26-55	±	±	±	±	±	±	—	—	—	—
26-56	±	±	±	—	—	—	—	—	—	—
26-58	±	±	±	±	—	—	—	—	—	—
26-59	+	—	±	±	—	—	—	—	—	—
			±	±	—	—	—	—	—	—

Group A — Intracutaneously sensitized; Group B — Intravenously immunized; Group C — Normal.

*Left eyes tested at this time.

low serum agglutinin titer was about the same in Groups A and B; but slight reactions were obtained with normal sera.

Experiment 2.—As summarized in Chart 2, the animals of Group A were sensitized as follows: After initial multiple inocula ranging from 10^{-1} cc. to 10^{-5} cc. of broth culture of Streptococcus V 110 A, a single dose of 10^{-3} cc. was given daily for 16 days; and on the 2nd, 7th, and 14th days the degree of sensitivity was tested with multiple inocula. The animals of Group B received the same doses of culture intravenously. The results of their comparative ophthalmic sensitivity and the agglutinin content of their blood sera on the 18th day is shown in Table III.

The rabbits in Group A were distinctly sensitive at this time, as indicated both by the cutaneous and ophthalmic reactions. Group B and Group C were not injected intracutaneously on this day; but the ophthalmic reaction performed in all three groups indicated decided sensitivity in Group A; two very slight reactions occurred in Group B, and none in Group C. The serum agglutinin titer was about the same in Groups A and B, and was distinctly increased in comparison with the normal group.

TABLE III

Experiment 2

Comparison of Groups on 18th Day

Rabbit No.	Cutaneous reaction	Ophthalmic reaction*	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	Control
26-91	Group A Intracutaneously sensitized ++++	++++	±	±	—	—	—	—	—	—
26-92	++±	++++	++	++	++	±	—	—	—	—
26-93	+++	++++	+	+	±	—	—	—	—	—
27-31	++++	++++	++	++	++	+	—	—	—	—
26-95	+++	++++	++	++	++	+±	+	±	—	—
26-96	++	+	+	—	—	—	—	—	—	—
26-97	+++	+++	+	±	±	—	—	—	—	—
26-98	Group B Intravenously immunized (Not tested this date)	±	++	++	++	+	+	±	—	—
26-99		—	++	+±	+	±	—	—	—	—
27-00		±	++	++	+	±	±	—	—	—
27-01		—	++	++	+±	+	—	—	—	—
27-02		—	++	±	±	—	—	—	—	—
27-03		—	++	++	++	+	—	—	—	—
27-04		—	++	++	++	+±	+	—	—	—
27-06	Group C Normal	—	±	—	—	—	—	—	—	—
27-08		—	—	—	—	—	—	—	—	—
27-09		—	—	—	—	—	—	—	—	—
27-05		—	—	—	—	—	—	—	—	—

* Right eyes tested at this time.

Subsequently small inocula were given only on the 23rd, 28th, and 34th days; but, as indicated in Chart 2, skin sensitivity increased in Group A until the 28th day, then fell off somewhat when comparative tests were repeated in all groups on the 44th day. The results are summarized in Chart 2 and Table IV.

The skin lesions were large and edematous in Group A, and much smaller in Groups B and C, with a slight, but distinctly greater and more nodular response in Group B than in the normal group (C). (In the latter group one

Effect of repeated small inocula of green streptococcus culture (Strain V 110 A) Group A (7 rabbits) intracutaneously, Group B (7 rabbits) intravenously, Group C (3 rabbits) normal.

CHART 2

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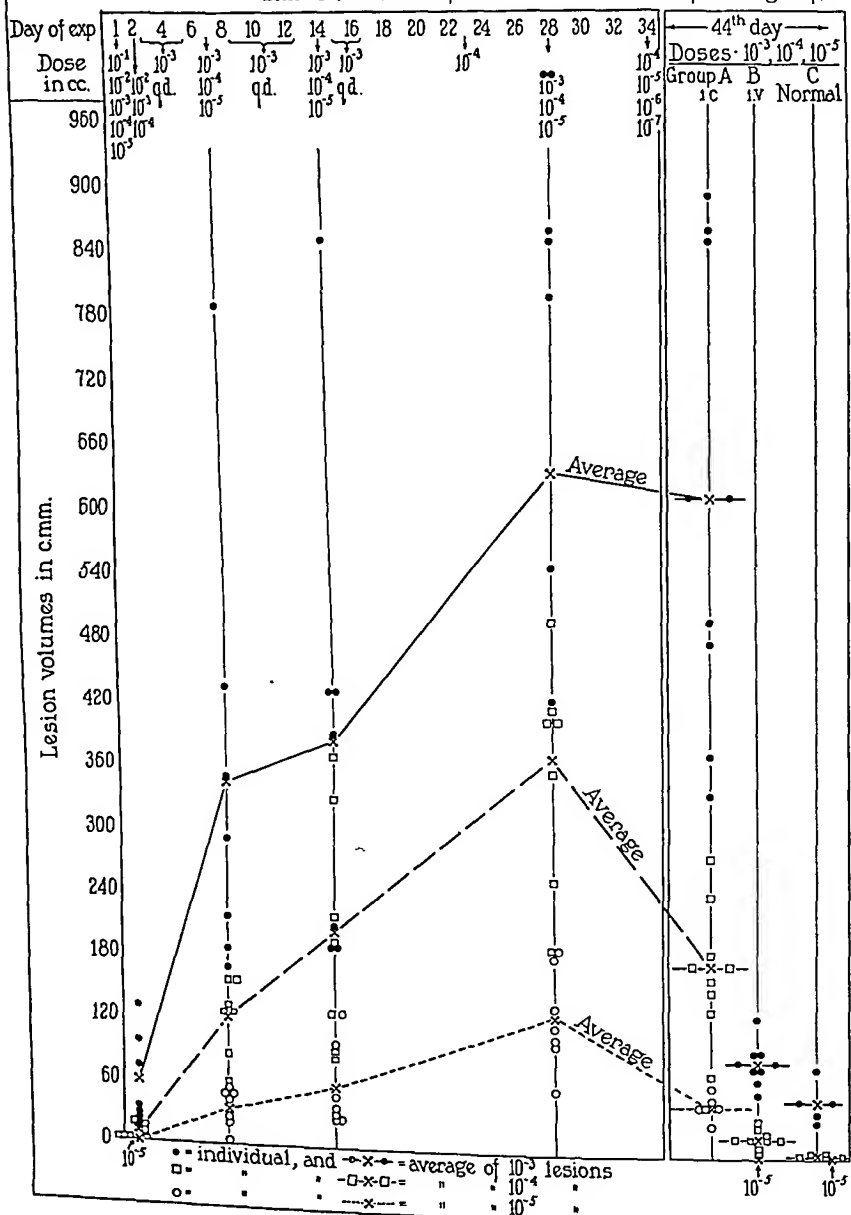
rabbit had died of an intercurrent infection, leaving only three for this last titration.) Again, a distinct increase in serum agglutinins was demonstrated in Groups A and B. Furthermore, the titer was, in general, higher in the intravenously inoculated group (B) than in the group receiving the injections intracutaneously (Group A), but the cutaneous reactions seemed independent of the variations in the antibody content of the sera.

TABLE IV

Experiment 2

Comparison of Groups on 44th Day

Rabbit No.	Cutaneous reaction		Agglutinations							
			1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	Control
26-91	Group A Intracutaneously sensitized	+++±	+	±	±	—	—	—	—	—
26-92		+±	++++	+++	+++	±	—	—	—	—
26-93		+±	+++	+	—	—	—	—	—	—
27-31		++++	++±	+++	++	+	—	—	—	—
26-95		+++	++±	++±	+++	+	±	±	—	—
26-96		+++	++	±	—	—	—	—	—	—
26-97		++++	++	++	+	—	—	—	—	—
26-98	Group B Intravenously immunized	±	++++	+++	+++	++±	+	±	—	—
26-99		++	++++	+++	++	+	±	—	—	—
27-00		±	++++	+++	+++	+	±	—	—	—
27-01		+	++++	++++	++±	++	+	±	—	—
27-02		±	+++	++	+	+	—	—	—	—
27-03		±	++++	++++	+++	++±	+	—	—	—
27-04		+	++±	++±	++±	++±	+±	++	—	—
27-06	Group C Normal	±?	±	±	—	—	—	—	—	—
27-08		±?	—	—	—	—	—	—	—	—
27-09		±	—	—	—	—	—	—	—	—

DISCUSSION

In spite of using antigens of such varying properties as vaccines prepared from virulent streptococci and living cultures of relatively avirulent green streptococci, the responses were in accord with one another and with previous observations. In the first experiment the total dose of antigen given in the form of vaccine over a period of 70 days was obtained from only 1.661 cc. of broth culture: in the second

a total 0.1349 cc. of living broth culture was administered within a period of 34 days. In spite of these relatively small inocula the animals, which had numerous areas of focal inflammation following intracutaneous inoculation, gave evidence of distinct hypersensitiveness. When, on the other hand, the route of preliminary inoculation was intravenous, and the animals gave no gross evidence of focal infection, the response to intracutaneous tests was much less than that of the intracutaneously inoculated groups, but slightly greater than that of normals. In a previous communication (3) it was noted that animals previously immunized with large doses of green streptococci responded to subsequent intracutaneous inoculation with lesions smaller than those of normal animals similarly tested, but this occurred only when the immunization was prolonged and intense; when, on the other hand, the immunization was short and mild such a diminution in response to inoculation was not so conspicuous. Some subsequent experiments have also indicated that the obtaining of a distinct state of hyporeactivity is dependent upon strong immunization. The results here presented, moreover, indicate that when the quantity of bacterial substance introduced intravenously is small and distributed over a long period, the resulting state of the tissues might possibly be designated as slightly hypersensitive when compared with that of normal animals; but it should again be noted that this degree of hypersensitiveness was much less than that of the animals which had suffered from multiple areas of focal inflammation. Another difference, not made evident by measurement alone, was constantly present: the lesions of the intravenously immunized animals were hard, nodular, and shotty on the 1st and 2nd days following intracutaneous inoculation, compared with small flat lesions in the normal animals and large edematous reactions in the cutaneously sensitized group. We regard the nodular type of response as indicative of the so called "hypoergic" immune state; and it is noteworthy that this qualitative type of reactivity was induced by such small intravenous inocula. It seems evident that only when the intravenous immunization is intense and prolonged is the quantitative "immune response" smaller than that of normals.

The evidence concerning the lack of quantitative relationship between agglutinin content of the blood serum and degree or type of

cutaneous response appears quite clear. Fortunately, the amount of bacterial substance given stimulated almost similar degrees of agglutinin formation in the two groups of animals receiving vaccines; and in the animals inoculated with living cultures of green streptococci the group injected intravenously had only slightly higher titer in the serum obtained on the 18th day than was present in the cutaneously sensitized group, but there was marked difference in the degree of hypersensitivity of the two groups. After an interval of 4 weeks, during which the animals received small inocula on only three occasions, the agglutinin titer of the serum from the intravenously immunized group was considerably enhanced, while that of the other group was only slightly raised; but as in previous experiments, no parallelism could be determined between the degree of hypersensitivity and the amount of agglutinin in the different animals. This, of course, does not prove a complete lack of relationship between antibody formation and hypersensitivity, because the antibody content of the tissues, in which the hyperergic reactions are made evident, is not necessarily reflected in the agglutinin content of the blood serum.

In human subjects Tuft (5) has recently observed fairly well marked cutaneous hypersensitiveness to be induced by intracutaneous injection of very small doses of horse serum; and Kohler and Heilman (6) demonstrated a slightly higher degree of cutaneous sensitization in children previously injected with small amounts of rabbit serum intracutaneously, than in those previously treated with similar doses intravenously. Such observations would make it appear that the cutaneous tissues respond in a similar manner to both intact bacteria and to soluble proteins. But the work of Opie (7) has demonstrated that with coagulable proteins the intensity of the Arthus phenomenon is roughly proportional to the antibody content of the serum against the antigen used for inducing the cutaneous reaction, a phenomenon not in accord with our findings with intact streptococci.

The size of the inocula approximated somewhat the amount of bacteria that may be active in human infections, or of vaccines used for treatment of patients. Comparative judgment of the relationship of dosage is difficult, however, because of the difference in size between rabbit and man. No attempt was made to increase the degree of immunity by doubling each successive dose; but rather an effort

was made to use the smallest dose for effective sensitization. These experiments, as well as others of a similar nature, indicate that the place in which the streptococci act in the animal body plays a very important rôle in the subsequent type of reactivity of the tissues, and that focal reactions are apparently more important factors in conditioning hypersensitiveness than is the antibody content of the blood serum.

SUMMARY

1. Rabbits were rendered very hypersensitive by relatively small doses of green streptococci given intracutaneously, and somewhat less hypersensitive by similar doses of heat-killed vaccine prepared from hemolytic streptococci.

2. Animals receiving the same doses intravenously gave, upon subsequent testing, lesions slightly more marked than normal controls; but these lesions were qualitatively hard and nodular compared with the large edematous lesions in the cutaneously sensitized group.

3. There was no parallelism between the degree of cutaneous or ophthalmic hypersensitivity and agglutinin titer in the blood serum.

4. Bacterial hypersensitivity to whole streptococci appears to depend more upon previously induced focal infection than upon circulating antibodies.

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I. RENAL THRESHOLDS FOR HEMOGLOBIN IN DOGS

DEPRESSION OF THRESHOLD DUE TO FREQUENT HEMOGLOBIN INJECTIONS AND RECOVERY DURING REST PERIODS

By JOHN A. LICHTY, JR., WILLIAM H. HAVILL, AND GEORGE H. WHIPPLE, M.D.

(*From the Department of Pathology, The University of Rochester School of Medicine and Dentistry, Rochester, N. Y.*)

For a number of years in this laboratory there has been an interest in the *conservation of hemoglobin* in experimental anemia. It would seem probable that the body would utilize every agency to conserve and reconstruct hemoglobin or hemoglobin building material under the conditions of a long continued anemia due to bleeding. It is very easy to demonstrate experimentally in dogs (8) that during prolonged anemia there will be very efficient conservation of hemoglobin introduced into the blood stream whether the blood hemoglobin comes from the dog, sheep or goose. In fact the introduced hemoglobin, to the extent of 90 to 100 per cent will be conserved (7) and rebuilt into finished red.cells which must be removed to maintain the fixed anemia level in the injected dog. It is obvious that the sheep and goose hemoglobin must be broken down to intermediates of unknown nature before the new dog hemoglobin and red blood cells are rebuilt. This observation reveals a very beautiful conservation of useful materials within the body.

The *renal threshold* obviously is related to this conservation of hemoglobin set free in the blood stream and a more complete understanding of the renal threshold for hemoglobin would be of especial interest to workers in this field. It was shown recently from this laboratory (3) that certain hemoglobins have widely different threshold values. In the normal dog the threshold for dog blood hemoglobin was determined as about 215 mg. hemoglobin per kilo body weight while the renal threshold for dog muscle hemoglobin was only about

11 to 15 mg. per kilo. Sheep and goose blood hemoglobin fell about midway between these extremes or 115 mg. per kilo.

Moreover the observation (5) that kidney feeding is almost as potent as liver feeding to produce new hemoglobin in experimental anemia due to bleeding, gives an added interest to a study of the kidney elimination and possible conservation of products coming from hemoglobin. Obviously materials are stored within the kidney which can be used to build up much new hemoglobin during long anemia periods in dogs. It is possible that some of these "building stones" are inherent in the individual cell protein of the kidney and have no relation to hemoglobin conservation but it is also possible if not probable that other "building stones" held in the kidney are related to hemoglobin breakdown and salvage within the body.

No more detailed exposition need be given to explain our continued interest in a study of renal thresholds for hemoglobin within the blood stream. It was decided to determine accurately the renal threshold for dog blood hemoglobin in normal dogs over long periods of time and from this base line to study variations which might be caused by agents injurious to the renal structures.

Our first surprise came when an attempt was made to establish a fixed base line for the hemoglobin threshold by daily injections of dog hemoglobin close to threshold values—sometimes a little above and alternately a little below. It became obvious that under these conditions with daily injection of hemoglobin the renal threshold was depressed. In other words the kidney filter seemed to become a less efficient barrier and let some hemoglobin through at a lower threshold value (Table 1). This renal threshold after subsiding to a certain level then became relatively constant if the frequent hemoglobin injections continued, but rose toward the initial higher level if the hemoglobin injections were discontinued for weeks or months (Charts B, C, D). This was all very confusing at first but experimental data given below will help toward a clearer understanding of these phenomena.

It will be observed that the threshold values for dog hemoglobin given by Manwell and Whipple (3) are somewhat higher than the initial values tabulated below. These differences are due to two factors, both related to the method used in these experiments. Manwell

and later Taylor used as their threshold a hemoglobin color in the urine recognizable by the eye although the color was always checked by the spectroscope. Urines which in gross were clear were recorded as negative and not examined by the spectroscope. In the experiments given below the urine was examined for hemoglobin bands as a routine whether it was grossly tinted or not and all specimens giving the characteristic bands were recorded as positive, although frequently grossly not colored. In other words the experiments below are based on a threshold of hemoglobin appearing in the urine in traces giving a micro spectroscopic but not necessarily a gross color test. This is probably more accurate and dependable.

The second factor is related to the *fading* of the hemoglobin color in the urine. Manwell and Taylor used a 3 hour collection period following the intravenous injection of hemoglobin, the dog being given water by stomach tube and placed in a metabolism cage. The experiments below are based on a $1\frac{1}{4}$ hour collection period, the urine being collected in a beaker or dish rather than in a cage. Small amounts of hemoglobin in the urine will fade or become invisible to the standard micro spectrometric test during an hour at room temperature or within the bladder.

These two differences in technique explain the differences in hemoglobin threshold values published by Manwell and Whipple (3) and tabulated below. We believe these later values to be more accurate and to approximate more nearly to the absolute renal threshold values. It does not seem necessary to review the literature as this was done in the first paper of this series (3).

Methods

Mongrel dogs were used in all experiments. These were of both sexes and ranged from 9 to 24 kilos in weight. They were kept in separate cages and fed on a diet of hospital scraps. Several control examinations of the urine of each dog were made to be sure that no detectable kidney damage was present before starting the series of experiments and checks were run at various intervals throughout their course. The clinical condition of the animals was normal at all times except where note is made to the contrary.

The dog blood hemoglobin used in practically all experiments was obtained from a colony of anemic dogs which were kept at a fairly low hemoglobin level by constant bleeding. It was prepared in a manner quite similar to that described

by Manwell and Whipple (3), the cells from the citrated blood being separated
washed twice with normal saline in a high speed centrifuge. They were t
laked with approximately twice their volume of distilled water by modera
severe shaking for at least 15 minutes. Following this the sediment was thr
down by centrifugalization at high speed for at least 30 minutes, and the c
supernatant fluid was decanted through several layers of cotton gauze. For
most part these solutions of hemoglobin were not made isotonic before inject
but in the few cases in which this was done, a sufficient amount of 10 per cent N
solution was added and the resulting precipitate removed as thoroughly as poss
by prolonged centrifugalization. Smith (6) has shown in blood volume stu
that considerable amounts of distilled water can be introduced intraveno
without causing hemolysis and our solutions were far below this amount and v
given slowly. The isotonic hemoglobin solutions obtained were in any event ne
as clear as the distilled water solutions of the hemoglobin pigment. Car
dioxide was never used to facilitate the separation of the stroma.

All hemoglobin solutions were immediately standardized by the acid hem;
method of Robscheit-Robbins (4). 1 cc. was diluted to 100 cc. with 1/10 N F
This solution was allowed to remain at least 1½ to 2 hours at room temperature ;
then compared with a standard solution of acid hematin. To avoid the erro
incomplete color development, the acid hematin solution was always left in
ice box overnight and a second comparison with the standard made.

For the injections a needle was carefully introduced into an external jugular v
and the injection mass (warmed to body temperature) allowed to flow in slo
from a standard 50 cc. burette. This method of injection was preferred to the
of syringes because it afforded greater uniformity of rate. Fresh hemoglobin v
used in all experiments—usually 3 to 4 hours after preparation of solution. I
mediately following this hemoglobin injection each dog (with a few exceptio
was given 250 to 500 cc. of warm tap water by stomach tube, the amount be
roughly proportional to the size of the dog. This was to minimize any kidr
damage produced by the hemoglobin (Barratt and York (2)) and to insure
early collection of urine. The dogs were fed once daily at about 4 p.m. and 1
injections were given in the forenoon or early afternoon, always before su
feedings.

In securing urine specimens the following procedure was a routine. In m
cases if the dog was removed from the metabolism cage 1¼ hours after injecti
a fresh, uncontaminated sample of urine could be obtained in a clean beaker
dish. In the few cases when dogs refused cooperation the sample was obtain
in the clean metabolism cage in which they were always placed following injecti
It was found that the majority of dogs would pass urine about 1 to 1½ hours af
the injection and this was adopted as the optimum time for obtaining the uri
Variations of 15 minutes on either side of this time were accepted, but experimer
with a greater variation in collection time were usually rejected, particularly
the urine showed no hemoglobin. Catheterization was never done because of t
danger of producing slight traumatic hemorrhage and cystitis.

When necessary the urine was filtered, but in any event it was tested for hemoglobin immediately using the ordinary Duboscq colorimeter with a Leitz micro spectrometer replacing the eye-piece. The presence of the definite absorption bands characteristic of oxyhemoglobin was accepted as the sole criterion for hemoglobinuria.

Fading of Hemoglobin in Urine

The urine of these dogs after collection of the usual $1\frac{1}{4}$ hour sample was always neutral to litmus or faintly alkaline, never strongly acid. If the urine gave positive bands for oxyhemoglobin in the spectroscope it was recorded as positive. It was noted that many positive specimens put aside for 2 to 3 hours at room temperature would subsequently give negative readings. The hemoglobin had "faded" and no longer gave the characteristic bands in the micro spectrometer.

This phenomenon was investigated further by observations on urine and dilute hemoglobin solutions set up in test tubes at room or incubator temperatures. A series of tubes containing 10 cc. normal dog urine was set up with increasing amounts of dog hemoglobin. After mixing the urine and hemoglobin, the solutions were read in the micro spectrometer and allowed to stand for some time, readings being made at intervals up to 5 to 6 hours at room temperature. It was found that in such mixtures of urine and dilute hemoglobin the fading amounted to approximately 1.5 mg. of hemoglobin per hour. At incubator temperature about twice as much fading would be recorded—that is the urine solution would lose about 3 mg. of hemoglobin each hour for 4 to 5 hours.

When the urine was boiled or when dilute solutions of phenol or formol were added to the mixture this fading was observed as usual. According to the observations of Baker and Dodds (1) the change from oxyhemoglobin to methemoglobin or acid hematin might be expected. We have made no effort to determine the end products but record the fading or disappearance of the oxyhemoglobin bands in urine mixtures.

We may record a typical experiment to illustrate the fading of hemoglobin within the bladder of a normal dog. A dog which had not been injected previously with hemoglobin was given intravenously a dose of 200 mg. dog hemoglobin per kilo body weight. Urine was retained in the bladder for 3 hours and when passed was negative for oxyhemoglobin bands. The same dose of dog hemoglobin was given the next day but the urine collected in a beaker at the end of $1\frac{1}{4}$ hours and this urine gave positive oxyhemoglobin bands. On the 3rd day the same dose of dog hemoglobin was given intravenously but the dog retained his urine for 5 hours. The sample was negative for oxyhemoglobin bands.

All this evidence makes it apparent that the urine in these experiments should be collected within $1\frac{1}{4}$ hours after injection and examined at once. Without doubt

there is a little fading even in this short time but by following a fixed routine this error can be held at a fairly constant level.

EXPERIMENTAL OBSERVATIONS

We use the term "renal threshold for hemoglobin" to indicate the smallest amount of hemoglobin which given intravenously will effect the appearance of recognizable hemoglobin in the urine. These values are given in terms of milligrams hemoglobin per kilo body

TABLE I

Depression of Renal Threshold for Hemoglobin Due to Frequent Injections of Dog Hemoglobin

Dog No.	Weight	Sex	Hemoglobin injections			Renal threshold for hemoglobin		
			Total	Urine Hb. +	Urine Hb. 0	Initial	Lowest	Decrease due to hemoglobin injection
	kg.					mg. per kg.	mg. per kg.	per cent
29-297	9.5	F	83	45	38	160	74	54
29-258	23.9	M	23	12	11	210	84	60
29-250	25.0	M	12	7	5	160	90	44
29-267	15.0	F	24	12	12	167	110	34
29-349	22.2	F	10	6	4	124	68	45
30-38	18.2	M	43	22	21	130	110	15
29-108	15.6	F	88	46	42	130	80	30
30-32	18.0	M	26	12	14	170	80	52
30-154	23.2	M	17	9	8	145*	60	59
Average........						155	84	46

* A recovery threshold—see Chart D.

weight. To determine this renal threshold we usually inject alternately subthreshold and superthreshold amounts of hemoglobin, beginning with calculated threshold values. Injections are usually made daily at about the same hour and the 24 hour interval is ample to permit escape of all injected hemoglobin from the circulating plasma.

Table 1 is a summary of injections given to each dog. The *initial renal threshold* is recorded for each dog as well as the *lowest renal threshold* which is observed after a considerable number of daily hemoglobin injections. On the average the renal threshold is depressed

about 46 per cent as the result of continued daily hemoglobin injections. The initial renal threshold shows considerable differences in different dogs from a maximum of 210 to a minimum of 124 mg. hemoglobin per kilo. We can offer no explanation for such differences but may say that dogs vary greatly in their physical capacity for work and this difference in renal threshold may in like manner depend on inherent functional differences in these dogs.
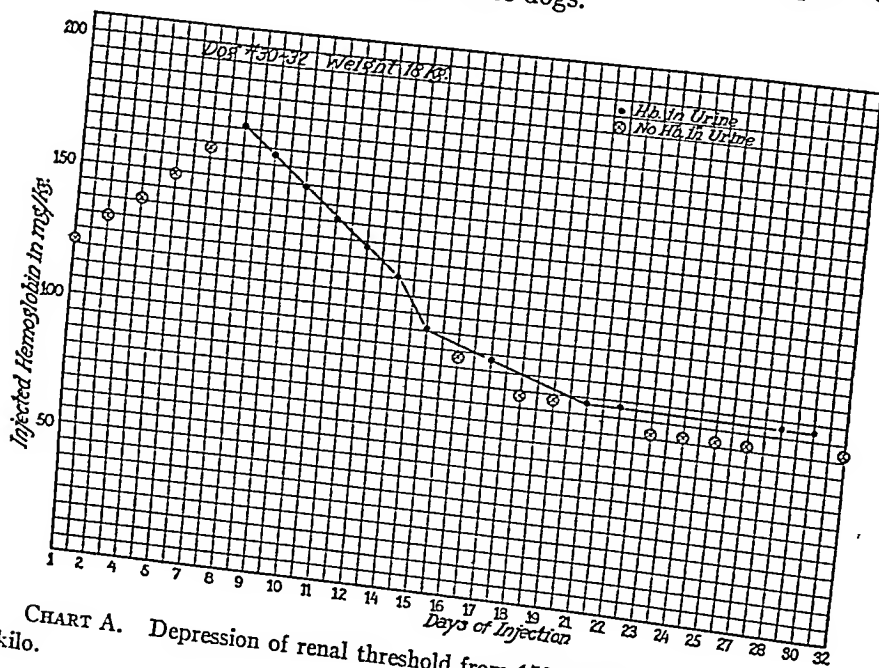
CHART A. Depression of renal threshold from 170 to 80 mg. hemoglobin per kilo.

The figures given for the *lowest renal thresholds* vary from a maximum of 110 to a minimum of 60 mg. hemoglobin per kilo but these values do not relate themselves to the values given for the initial thresholds. A dog having a high initial threshold may show a depressed threshold which is lower than the average (Dog 29-297, Table 1). A dog with a low initial threshold may show only a slight depression of that threshold and therefore a depression threshold that is above the average level (Dog 30-38, Table 1).

Many observations on hemoglobin injections in other dogs have

been made in the course of this study but it did not seem necessary
to record these experiments some of which are incomplete in one
respect or another.   These unrecorded experiments are completely
in harmony with those recorded in these papers and give added support
to the published experiments.   No differences were observed when
isotonic solutions of hemoglobin were used.   No differences were
recorded when all water was withheld during an experiment and we
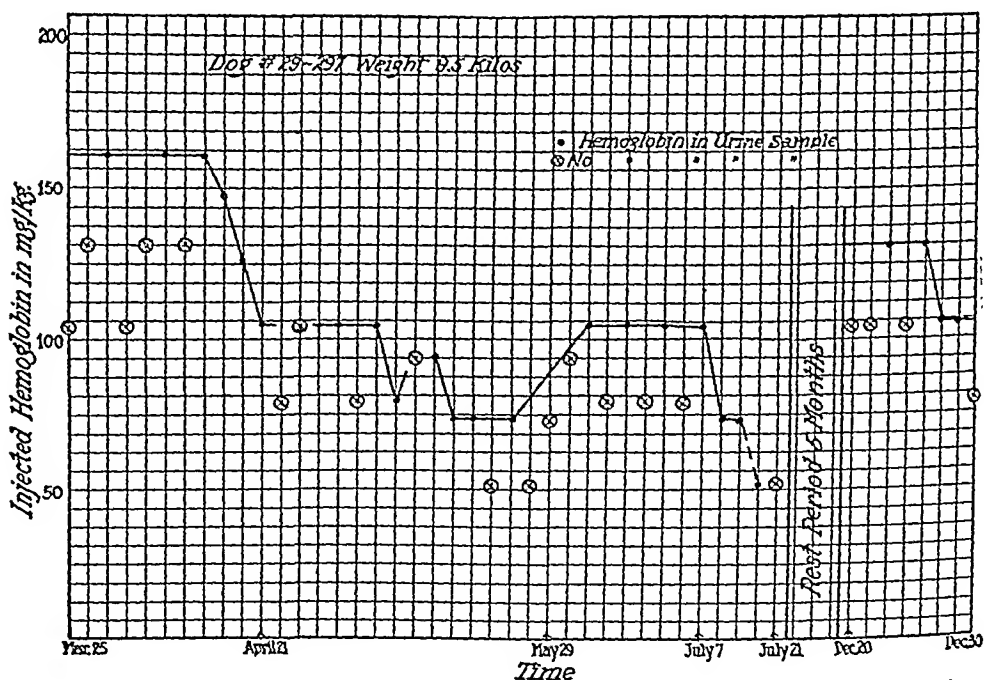
CHART B.   Gradual depression of threshold and recovery during rest period.

have no evidence that moderate diuresis has any influence on the renal
threshold for hemoglobin.

For sake of condensation in Charts A, B, C and D all the injections
are not recorded.   The black lines connecting the positive values are
used to aid the observer in appreciating the approximate depression
of the true renal threshold for dog hemoglobin in dogs.

Chart A shows a rapid drop in the renal threshold for hemoglobin
between the 8th and 16th days of injection.   If the initial injection
had been higher, for example 180 to 190 mg. per kilo, it is possible
that there would have been no escape of hemoglobin in the urine at

this higher level.   The final level is quite satisfactorily established with a series of injections close to the threshold, some subthreshold and others superthreshold.

Chart B shows a long series of experimental injections.   Many given the animal are not included in this chart.   There is an irregular slow depression of the renal threshold for hemoglobin.   Injections were given several times every week with occasional short rest inter-
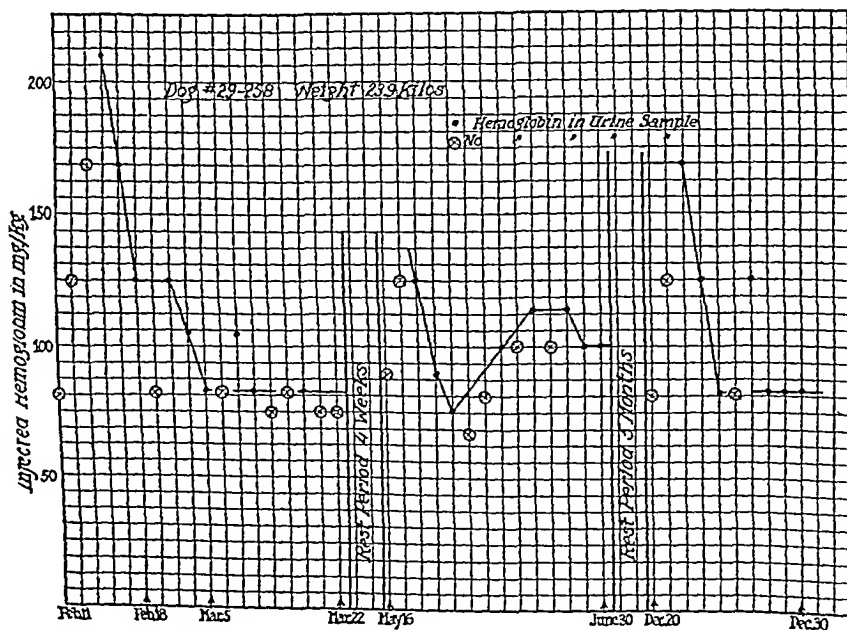
CHART C.   Sharp depression of renal threshold and moderate recovery during rest periods.

vals throughout the 4 months preceding the rest period.   There is a definite recovery toward the original threshold value during the 5 months rest period and then we observe a fall in threshold values as the hemoglobin injections are continued during December.   During rest periods the dogs were given no injections (Charts B, C and D) but were kept in cages and fed a mixed diet of hospital scraps.

Chart C shows a marked depression in the renal threshold for hemoglobin within a month and a moderate degree of recovery during rest

periods but the recovery thresholds are never as high as the origina
threshold. The depression threshold or minimal levels are reasonably
uniform at about 85 to 100 mg. hemoglobin per kilo.

Chart D brings out an interesting point. A considerable series of
subthreshold injections (100 mg. hemoglobin per kilo) were given and
eventually the renal threshold was depressed by this procedure to a
level below 100 mg. per kilo. A rest period of 16 days showed a
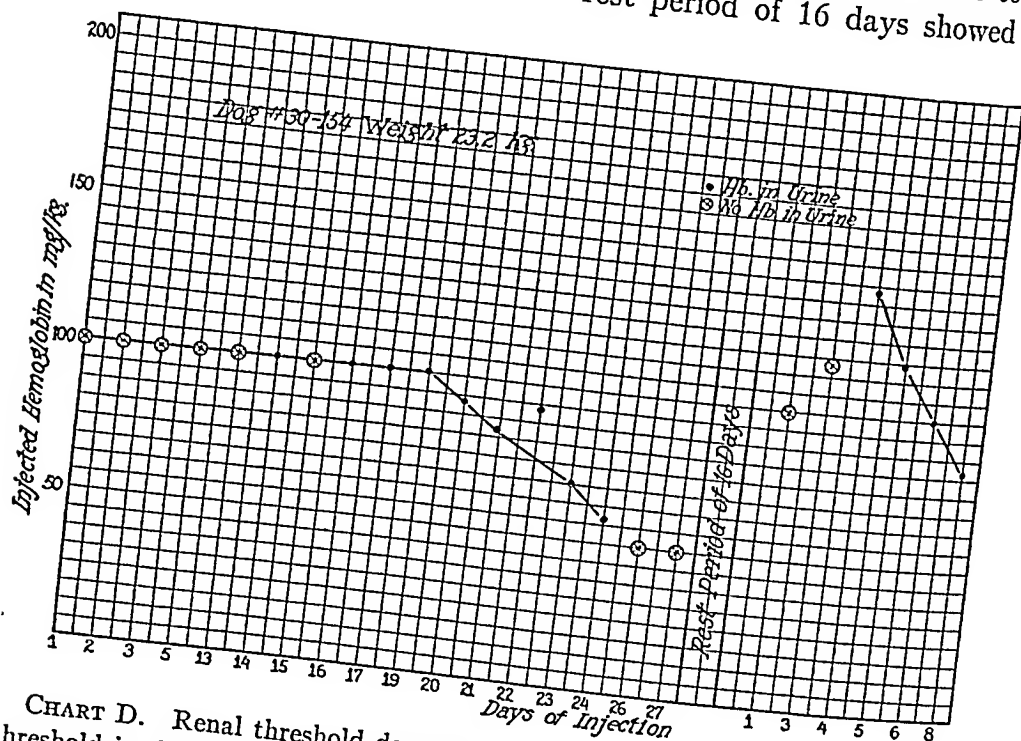
CHART D. Renal threshold depressed by subthreshold injections. Recovery
threshold is above original injections level.

recovery threshold of 145 mg. per kilo which we may be sure is below
the original threshold if we had determined it in the usual way.

This type of experiment has an important bearing upon our concep-
tion of the mechanism of the renal threshold for hemoglobin. Our
first thought was that a supertheshold dose of hemoglobin might cause
slight injury of the renal filter with increased permeability and lower-
ing of threshold values. This experiment (Chart D) shows that a
series of subthreshold injections may eventually decrease the renal
threshold so that hemoglobin appears in the urine. The histological

findings (Paper IV) and iron analyses (Paper V) given below show that the *tubular renal epithelium* is responsible for this reaction.

Experiments with sheep hemoglobin have been performed but have not been carried out in sufficient detail to warrant publication at this time. It is probable however that sheep hemoglobin behaves like dog hemoglobin in experiments dealing with renal threshold values.

DISCUSSION

For the sake of clarity it may be worth while to outline our conception of the renal threshold for dog hemoglobin and its various fluctuations due to repeated intravenous injections of hemoglobin. This concept applies only to dog blood hemoglobin and its renal threshold in the normal dog kidney. We speak of the *initial renal threshold* by which term we mean the least amount of hemoglobin which early in the experiment will give a recognizable escape of hemoglobin into the urine—this figure averages in these experiments about 155 mg. hemoglobin per kilo body weight. As injections of hemoglobin are given daily we note a *depression of the renal threshold* which may show an average decrease of 46 per cent below the initial threshold. With rest periods there is a tendency for the threshold to return toward the initial level and we may term this the *recovery threshold*.

The lowest renal threshold or *depression threshold* reached only after many repeated hemoglobin injections is fairly constant and we believe this approximates the *glomerular threshold* by which term we mean the standard level of hemoglobin in the blood plasma which will cause escape of hemoglobin into the glomerular capsule. This glomerular threshold may be expressed in terms of milligrams of hemoglobin per kilo body weight and presumably is a little below the lowest or depression threshold which in our experiments (Table 1) averages about 84 mg. hemoglobin per kilo body weight.

This term *glomerular threshold* indicates our belief that in these experiments the hemoglobin escapes through the glomerular tuft whose cells act as a barrier and establish the absolute renal threshold for dog hemoglobin. The evidence for our belief is scattered through this group of papers but it seemed best to outline this thesis and the experimental data will fall into place as the argument proceeds.

From evidence above and in Paper IV it would appear that when

hemoglobin first escapes into the renal tubules it is rapidly take
and in part at least deposited within the tubular epithelium.   A
process continues with repeated daily injections the epithelium
take up less and less hemoglobin, therefore the renal thresho
bladder urine falls or approaches the glomerular threshold.

From evidence in Paper II we note that the lowest or *depr*
renal threshold is not modified by moderate renal injury due to mer
chloride poisoning which is known to injure almost specially
tubular epithelium of the kidney.   It seems difficult to explain
unchanged renal threshold for hemoglobin after bichloride inju
the kidney if it is assumed that the dog hemoglobin passes throug
tubular epithelium into the lumen of the tubules.

From evidence in Paper IV it is seen that doses of hemog
definitely below the glomerular threshold although given over
periods of time never cause any deposit of iron containing pig
within the tubular epithelium of the kidney, although all the c
organs are pigmented exactly as though a superthreshold seri
hemoglobin injections had been given.

One can deplete the renal tubular epithelium of its pigment,
and probably other stored intermediates related to hemoglobi
periods of simple anemia due to bleeding.   This is further eviden
Papers IV and V—that renal epithelium has to do with conserva
of hemoglobin factors under certain conditions.

SUMMARY

We use the term "renal threshold for hemoglobin" to indicate
smallest amount of hemoglobin which given intravenously will e
the appearance of recognizable hemoglobin in the urine.

The *initial* renal threshold level for dog hemoglobin is establis
by the methods employed at an average value of 155 mg. hemogl
per kilo body weight with maximal values of 210 and minimal of 12

Repeated daily injections of hemoglobin will depress this in
renal threshold level on the average 46 per cent with maximal va
of 110 and minimal values of 60 mg. hemoglobin per kilo body wei
This minimal or *depression threshold* is relatively constant if the in
tions are continued.

Rest periods without injections cause a return of the renal thresh

for hemoglobin toward the initial threshold levels—a *recovery threshold* level.

Injections of hemoglobin below the initial threshold level but above the minimal or depression threshold will eventually reduce the renal threshold for hemoglobin to its depression threshold level.

We believe the *depression threshold* or minimal renal threshold level due to repeated hemoglobin injections is a little above the *glomerular threshold* which we assume is the base line threshold for hemoglobin. Our reasons for this belief in the glomerular threshold are given above and in the other papers of this series.

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II. RENAL THRESHOLD FOR HEMOGLOBIN IN DOGS UNINFLUENCED BY MERCURY POISONING

By WILLIAM H. HAVILL, JOHN A. LICHTY, JR., GORDON B. TAYLOR, M.D., AND GEORGE H. WHIPPLE, M.D.

(From the Department of Pathology, The University of Rochester School of Medicine and Dentistry, Rochester, N. Y.)

The first paper submits evidence to show that the initial renal threshold for hemoglobin is depressed by frequently repeated injections of hemoglobin. This depression or minimal threshold is reasonably constant if the hemoglobin injections are continued daily but the renal threshold rises toward the initial threshold level during rest periods with no hemoglobin injections.

The *minimal or depression threshold* for dog hemoglobin is not changed by moderate doses of mercuric chloride given intravenously. Under such conditions there is ample evidence of injury of the renal tubular epithelium but no evidence of glomerular injury. The experiments tabulated below are difficult to explain if we postulate secretory activity on the part of the tubular epithelium related to the escape of hemoglobin from the blood plasma into the urine. The evidence fits much better with the assumption that the hemoglobin passes the glomerular filter and may be absorbed by the tubular epithelium unless that epithelium is injured by mercury or stuffed with previously absorbed hemoglobin coming from many preceding hemoglobin injections. This argument is given further support by experimental data tabulated in Papers IV and V below.

Experimental hemoglobinuria has not been neglected by investigators during the past 30 years and one can assemble evidence to the effect that the hemoglobin passes only through the glomeruli (Ribbert (10), Baker and Dodds (1)), that it is secreted only by the tubules (Miller (9), Lehnert (5)) or that it is filtered through the glomeruli with additions made by the tubular epithelium (Fukuda and Oliver (3)). There have been so many excellent reviews of this whole ques-

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tion during recent years that it seems unnecessary to cover this subject again.

The question of the toxicity of mercuric chloride for dogs and the character of the renal lesions have been reviewed by Sansum (11), Haskell, Hamilton and Henderson (4), and MacNider (6). The evidence is overwhelming that the injury of tubular epithelium is the conspicuous feature.

EXPERIMENTAL OBSERVATIONS

The methods were identical with those described in the preceding paper, in fact some of the same dogs were used in both types of experiment. The mercuric

TABLE 21

Renal Threshold for Hemoglobin Uninfluenced by Mercury Poisoning

26 hemoglobin injections given before this experiment

Lowest normal or depression renal threshold for hemoglobin = 80 mg. per kilo

Dog 30-32, male, weight 18 kilos

Experiment, days......	1	2	3	4	5	7	8	12	13	14	15	17	18	19	20	21	28
Urine albumen.....	0	Tr.	Tr.	2+	Tr.	Tr.	0	3+	2+	2+	+	Tr.	0	0	0	0	0
$HgCl_2$, mg. per kg...	1	0	0	0	0	0	0	1.5	0				0	0	0	0	3*
Hb. intraven., mg. per kg..........	0	70	70	70	70	70	80	0	70	130	70	80	90	80	80	80	0
Hb. in urine.......	0	0	0	0	0	0	+	0	0	+	0	0	+	+	+	+	0

* Death 30 minutes after mercury injection and autopsy at once.

chloride was given intravenously dissolved in 150 to 200 cc. of a 10 per cent glucose solution, the solution flowing in slowly by gravity. Giving the mercuric chloride intravenously has many advantages over administration by mouth which may be complicated with vomiting and gastro-enteritis. Occasionally one will produce death within a few hours by this intravenous method and the general picture is that of shock due to colloid injection. Fresh urine samples were examined for albumen and formed elements the day before injection and each day thereafter for 5 days or longer. As the albumen seems to be the most constant index of this type of acute renal injury we record in the tables only data relating to the albuminuria.

Table 21[1] gives satisfactory data to show that the minimum or depression renal threshold for dog hemoglobin is not changed by

[1] The number borne by this table and the later ones is indicative of the paper from which it comes as well as its order in the paper. Thus for example Table 21 is the first table of Paper II, Table 34 the fourth table of Paper III.

moderate injury of the kidney produced by intravenous injection of mercuric chloride in moderate doses.

The clinical condition of the dog was normal up to the day of lethal reaction to the last injection of mercury. He was active and ate food as usual. The urine however gave clear evidence of injury following both the 1 mg. and 1.5 mg. per kilo doses of mercuric chloride. With the presence of albumen we always record the finding of epithelium and granular casts. No red blood cells were found. From the control animals and others killed soon after a sublethal dose we may be sure that there was injury of the tubular epithelium which however was promptly repaired. It is obvious that the lowest or depression threshold of 80 mg. per kilo established in the weeks preceding this experiment (Chart A, Paper I) is not modified by the injury of tubular epithelium. The threshold is very constant and obviously is very close to 80 mg. per kilo as in one instance this dosage gives a negative urine. That the threshold is not lowered even slightly is shown by the frequent injections of 70 mg. per kilo which are invariably negative.

The autopsy on this dog (Table 21) was done at once after death from intravenous injection. It is obvious that this reaction is not related to the kidneys but to the body as a whole and comprehends whatever the reader carries in his mind as related to peptone shock. The autopsy findings were of no interest to this experiment except the kidneys which in gross were practically normal except for congestion.

Histological sections (Dog 30-32, Table 21) show a normal picture for glomeruli and tubules in almost all fields. The glomeruli show no evidences of injury. A few tubules show occasional desquamated epithelial cells and cell casts which obviously relate to the mercuric chloride injection of 1.5 mg. per kilo given 16 days before death. The repair of the mercury injury however is practically complete and perfect. The pigment in the tubular epithelium is not abundant in this case and this is probably explained by the relatively small number of superthreshold hemoglobin injections which preceded the autopsy. These kidneys show occasional clusters of mononuclear cells and small scars in various parts of the cortex which may include an occasional abnormal glomerulus or tubule. This may indicate some focus of injury or infection but we find these areas in practically all stock dogs of middle age and there is a tendency toward increase with age.

Table 22 shows results identical with Table 21 but this dog tolerated very large doses of mercuric chloride.

The minimal or depression renal threshold was well established at 70 mg. hemoglobin per kilo by a long series of injections preceding these experiments. Attention was directed particularly toward the *lower threshold levels* so that repeated injections of 53 mg. hemoglobin per kilo were done over and over again always with a negative urine in spite of large doses of mercuric chloride. In our experience

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the control dog usually dies after a 2 mg. mercuric chloride per kilo dose (Tab[le] below) but this dog tolerated 4 mg. mercuric chloride per kilo with but mode[rate] injury of kidney epithelium. This point is taken up again in Paper III. The [...] of 66 mg. hemoglobin per kilo is positive and practically coincides with the [...] pression threshold of 70 mg. per kilo. We do not believe it is possible to estab[lish] renal threshold closer than 5 mg. hemoglobin per kilo and often the individu[al] will show a fluctuation in this threshold of 10 mg. hemoglobin per kilo.

This dog was clinically normal throughout the experiment up to the time of th[e] injection which caused death within an hour with symptoms of shock. It i[s] obvious that this last large dose of 8 mg. mercuric chloride per kilo had no time to cause histological changes in the kidney so that this organ will give a satisfactory picture of the injury done by the preceding dose of 4 mg. mercuric chloride per kilo given 5 days before death. The autopsy findings are of no interest for this experi-

TABLE 22
Renal Threshold for Hemoglobin Uninfluenced by Mercury Poisoning
85 hemoglobin injections given before this experiment
Lowest normal or depression renal threshold for hemoglobin = 70 mg. per kilo
Dog 29-198, male, weight 22.7 kilos

Experiment, days	1	2	3	4	5	6	18	19	20	21	25	26	27	28	31
Urine albumen	0	0	0	—	Tr.	Tr.	0	Tr.	Tr.	+	+	+	2+	Tr.	—
$HgCl_2$, mg. per kg.	1.25	0	0	1.4	0	0	2.0	0	0	2.5	0	4.0	0	0	8.0*
Hb. intraven., mg. per kg.	0	0	50	53	0	0	66	53	0	0	53	0	53	0	0
Hb. in urine	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0

* Death 1 hour after injection and autopsy at once.

ment except for the kidneys which in gross were practically normal except for moderate pigmentation.

Histological sections (Dog 29-198, Table 22) show some definite evidence of injury and repair but the great bulk of the tubules present a normal appearance. Pigment is abundant in the epithelium of most of the convoluted tubules and represents iron containing residues derived from the injected hemoglobin. This subject is discussed in detail in Paper IV. The glomerular tufts are all normal. A few tubules show evidence of epithelial regeneration and mitotic figures. Occasional cell casts are noted. The mercury obviously caused no injury to glomeruli and only moderate injury to the tubules where some epithelium was destroyed. Other tubular epithelium cells were slightly injured but were repaired during the 5 days which elapsed between the 5th mercury injection and death from a lethal injection.

Table 23 shows an experiment much like the two preceding ones but particular attention was given to threshold values just above the minimal or depression threshold levels.

For the previous history and hemoglobin injection consult Table 1 and Chart D, Dog 30-154, Paper I. It gives clear evidence that there is no rise of the threshold values except in the very last observation when an injection of 90 mg. hemoglobin per kilo gives a negative urine. The dog died a few days later as the result of renal injury due to 3 mg. mercuric chloride per kilo and these kidneys showed extreme epithelial necrosis which blocked completely the lumina of many tubules. It would seem that this extreme change would give adequate explanation for this single negative observation with a superthreshold dose. We believe that this is purely a mechanical factor which is present only when extreme tubular injury and obstruction is caused by the mercury. Anuria is the next stage which often supervenes in these lethal cases.

This dog 30-154 (Table 23) tolerated small doses of mercuric chloride very well and showed little evidence of renal injury even to a large dose of 2.5 mg. mercuric chloride per kilo. There was no clinical evidence of renal disturbance up to the last dose of 3 mg. mercuric chloride per kilo when some diarrhoea and vomiting appeared shortly before death. The dog died 8 days following the last large dose of mercury and presented all the evidences of renal insufficiency. Autopsy was done promptly after death. The findings are of no interest to this experiment except for the kidneys which were surprisingly normal in gross. Histological sections show a typical picture of *mercuric chloride nephrosis*. The majority of the tubules show necrotic epithelium which in many causes complete blockage of the tubules. Some of this necrotic material is impregnated with lime salts giving the tissue a deep blue granular appearance. The glomerular tufts are all normal. Some tubules have escaped the necrosis and show the usual yellow granular pigment due to the hemoglobin injections. Casts are everywhere numerous. Some efforts to repair the tubular epithelium are noted with proliferation of new epithelium.

Table 24 is much like the preceding experiments and this dog tolerated large doses of mercury with minimal evidence of renal injury. During this time there was no change in the lowest renal threshold values.

A single dose of 100 mg. hemoglobin per kilo gave a negative urine but all other like doses showed a positive urine. This indicates that the 100 mg. hemoglobin level was very close indeed to the absolute renal threshold. The last dose of 2.5 mg. mercuric chloride per kilo gave practically no evidence of renal injury as there was no albuminuria.

Following this last experiment this dog was not injected with any mercury nor

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TABLE 23

Renal Threshold for Hemoglobin Uninfluenced by Mercury Poisoning

17 hemoglobin injections given before this experiment

Lowest normal or depression renal threshold for hemoglobin = 60 mg. per kilo

Dog 30-154, male, weight 23.2 kilos

Experiment, days	1	2	3	5	12	15	16	17	18	19	22	23	24	26	37	38	40	43	44	45
Urine albumen	0	0	0	0	0	0	0	0	Tr.	0	0	+	0	0	0	3+	3+	3+	3+	
$HgCl_2$, mg. per kg	1.25	0	0	0	0	1.75	0	0	0	0	2.5	0	0	0	3.0	0	0	0	0	
Hb. intraven., mg. per kg	0	80	50	100	120	0	120	120	90	90	0	0	90	100	0	0	90	0	0	
Hb. in urine	0	+	0	+	2+	0	2+	+	+	+	0	0	+	+	0	0	0	0	0	
Clinical condition	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	D	D V	D V	*

N = normal, D = diarrhoea, V = vomiting.

* Death and autopsy at once.

TABLE 24

Renal Threshold for Hemoglobin Uninfluenced by Mercury Poisoning

83 hemoglobin injections given before this experiment

Lowest normal or depression renal threshold for hemoglobin = 100 mg. per kilo

Dog 29-297, female, weight 10.2 kilos

Experiment, days	1	2	3	4	8	10	11	12	13	14	15	17	18	19	20	21	36	37	38	39
Urine albumen	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Tr.	0	0	0	0	0
$HgCl_2$, mg. per kg	1	0	0	0	0	1.5	0	0	0	0	0	2	0	0	0	0	2.5	0	0	0
Hb. intraven., mg. per kg	0	80	80	80	150	0	0	100	80	150	100	0	150	100	0	0	0	80	80	100
Hb. in urine	0	0	0	0	2+	0	0	+	0	2+	+	0	2+	0	0	0	0	0	0	+

given hemoglobin for 3.5 months but was bled about twice a week 100 to 200 cc. whole blood which reduced her red cell hematocrit from the initial level of 42 per cent to a level ranging from 35 to 25 per cent. This moderate grade of anemia was produced to note whether the iron containing pigment was removed from the renal tubular epithelium and whether the kidneys subsequently were less resistant to mercury poisoning. After 3.5 months this dog was given an identical dose of mercuric chloride 2.5 mg. per kilo which previously had caused no disturbance. This second dose caused a decided albuminuria but not a lethal injury. Five days later the dog was in good clinical condition and the urine had returned to normal. She was given gas and killed and the autopsy done at once. The anatomical findings are of no interest except for the kidneys which were practically normal in gross.

Histological sections (Dog 29-297, Table 24) show a renal picture which could hardly be differentiated from a normal stock dog. The glomeruli and tubules are normal so that evidently the slight injury had been completely repaired. A very few tubules show in their epithelium a few tiny yellow pigment grains but practically all of the deposited pigment was removed during this rest period combined with a moderate anemia. This point will be referred to again in Papers IV and V.

This dog was somewhat more resistant to mercuric chloride than control dogs even after she had been made anemic to remove almost all the pigment in the tubular epithelium. Whether the preceding series of mercury injections was a factor we cannot say—refer to MacNider's (7) work with uranium and increased tolerance following a previous injury.

DISCUSSION

We do not propose to plunge into the troubled waters of debate relating to the secretory mechanism of the kidney glomeruli and tubules. We refer merely to the admirable papers of MacNider (6), Richards (12), Edwards (2), Marshall (8) and many others. We will attempt to limit our discussion to the behaviour of dog hemoglobin when it is eliminated from the blood plasma into the urine. Possibly some of the confusion in the literature comes from an attempt to correlate findings in cold blooded animals with those in warm blooded animals. It surely is possible that the mechanism of urinary secretion may differ in the frog as compared with the dog, just as it must be different in some of the primitive fishes which possess no glomeruli within their kidneys.

The experimental evidence tabulated above is strongly supported by Tables 31, 32 and 36 in Paper III. The minimal or *depression*

renal threshold is evidently not modified by moderate doses of mercuric chloride and consequent injury done the renal tubules. The same is true even for somewhat severe renal tubular injury, short of actual obstruction to the flow through the tubules. There is no evidence for glomerular injury. We believe this means that from the blood plasma the injected hemoglobin passes out through the glomerular tuft into the convoluted tubules. It passes through these tubules unchanged if there have been frequent preceding hemoglobin injections even if the tubular epithelium has been injured by mercury injections. The normal kidney before it has been subjected to frequent hemoglobin injections will take up much hemoglobin from the tubule into the tubular epithelium and this explains the high initial renal threshold for hemoglobin and its subsequent depression to a relatively fixed or minimal threshold. It is not surprising that this tubular epithelium reaches a repletion stage beyond which it can take in no more pigment material until it has disposed of this pigment and other related substances according to its individual method.

We are unable to explain our findings if the renal tubular epithelium in the dog participates actively in the passage of dog hemoglobin out of the plasma into the tubular lumen.

We believe that this minimum or depression renal threshold comes quite close to the absolute base line or glomerular threshold. The picking up of hemoglobin from the tubular lumen is an important factor in conservation of hemoglobin and hemoglobin "building stones."

SUMMARY

The minimal or depression renal threshold for dog hemoglobin is not modified by moderate doses of mercuric chloride.

This type of renal injury involves the epithelium of the convoluted tubules but the glomeruli escape.

We are unable to explain our findings if we assume that the tubular epithelium takes an active part in the passage of dog hemoglobin from the blood into the urine.

The evidence points toward the glomerular tuft as responsible for the passage of the hemoglobin from the blood plasma into the tubules. The glomerular tuft establishes the true hemoglobin threshold under these conditions.

If the convoluted tubules are normal, we note that hemoglobin is taken into the epithelium and this explains the high initial renal threshold.

With repeated hemoglobin injections this tubular epithelium becomes stuffed with hemoglobin pigment fractions and can absorb no more, which explains the minimal or depression threshold. Further injury of this tubular epithelium with mercury causes no change in this minimal renal threshold, unless we produce actual tubular obstruction.

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III. TOLERANCE FOR MERCURY POISONING INCREASED BY FREQUENT HEMOGLOBIN INJECTIONS

By WILLIAM H. HAVILL, JOHN A. LICHTY, Jr., AND GEORGE H. WHIPPLE, M.D.

(From the Department of Pathology, The University of Rochester School of Medicine and Dentistry, Rochester, N. Y.)

When we began to compare the effects of mercuric chloride poisoning in a series of dogs receiving frequent intravenous hemoglobin injections with a series of control dogs it was obvious that the hemoglobin series tolerated about twice as much mercury as did the controls. This degree of tolerance is not extreme but it is of some interest in view of the histological findings and is perhaps related to the tolerance for uranium observed in dogs by MacNider (2). Given repeated intravenous superthreshold doses of hemoglobin in the dog we observe the deposit of yellow granular pigment within the epithelium of the convoluted tubules—see Paper IV. This pigment gives an iron stain and is obviously related to the injected hemoglobin. Beyond a certain point this epithelium cannot take up any more of this pigment material. This material perhaps or something related to it, renders the epithelium somewhat resistant to injections of mercuric chloride but does not protect against very large doses of mercury.

EXPERIMENTAL OBSERVATIONS

The same methods are employed in all these experiments as have been described in the preceding papers. We refer to Tables 22, 23 and 24 in Paper II as they give evidence of value to show an increased tolerance for mercury in dogs which had received numerous hemoglobin injections. Table 22 is of especial interest as this dog tolerated a dose of mercuric chloride of 4 mg. per kilo with but slight injury to the tubular epithelium. Table 24 also shows a dog which tolerated a dose of 2.5 mg. per kilo without evidence of any tubular injury and with no albuminuria. In our experience with control stock dogs (no

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hemoglobin injections) a dose of mercuric chloride of 1.5 or 2.0 mg. per kilo is fatal with the typical picture of a mercury nephrosis (Tables 33 and 34).

Table 31 presents average findings. This dog had received 43 hemoglobin injections previous to this experiment and we may be sure had abundant iron containing pigment in the renal tubular epithelium. The dog tolerated 1.0 and 1.5 mg. mercuric chloride per kilo without any clinical disturbance. The renal threshold values relate to Paper II. The last injection of 3.0 mg. per kilo caused death only after 8

TABLE 31

Resistance of Renal Epithelium to Mercury Poisoning Increased by Frequent Hemoglobin Injections

43 hemoglobin injections given before this experiment

Lowest normal or depression renal threshold = 110 mg. per kilo

Dog 30-38, male, weight 18.2 kilos

Experiment, days	1	2	3	4	5	7	8	9	10	11	14	15	16	17	18	19	22
Urine albumen	0	Tr.	0	0	0	0	0	0	0	0	0	3+	4+	4+	3+	3+	
$HgCl_2$, mg. per kg	1	0	0	0	0	1.5	0	0	0	0	3	0	0	0	0	0	
Hb. intraven., mg. per kg	0	120	90	90	90	0	90	90	0	120	0	90	90	90	90	90	
Hb. in urine	0	+	0	0	0	0	0	0	0	+	0	0	0	0	0	0	
Clinical condition	N	N	N	N	N	V	N	D	N	N	N	V	D/V	V	D/V	S	*

N = normal, V = vomiting, D = diarrhoea, S = intoxicated.
* Death and autopsy at once.

days and this dose was surely a minimum lethal dose for this dog. He came very close to recovery and reparative processes were active in the kidney at time of autopsy.

Dog 30-38, Table 31. Dog died 8 days after a large dose (3 mg. per kilo) of mercuric chloride and autopsy was done at once. The usual stomatitis was present but there were no lesions of the gastrointestinal tract either gross or microscopic. The findings are of no interest for this experiment except the kidneys which appear grossly normal. *Histological sections* show that this injury was minimal to cause death and this dog came very close to recovery. The amount of massive hyaline necrosis of convoluted tubular epithelium is considerable but not compara-

ble to the controls. Very many convoluted tubules show active repair. Some tubular epithelium shows much fat. Hyaline casts are fairly numerous and some of these hyaline areas contain calcium salts which give a dark blue stain. Pigment is moderate in amount and is found in necrotic epithelium and hyaline material as well as in residual epithelium. The new formed epithelium obviously contains no pigment. The glomerular tufts are normal. The collecting tubules show casts but normal epithelium.

A control experiment was done at the same time under identical conditions. Dog 29-114, Table 33. Dog died 3 days after the second small dose (1.5 mg. per kilo) of mercuric chloride and was autopsied at once. The kidneys were relatively normal in gross. *Histological sections* present the usual picture of a bichloride

TABLE 32

Resistance of Renal Epithelium to Mercury Poisoning Increased by Frequent Hemoglobin Injections

88 hemoglobin injections given before this experiment

Lowest normal or depression renal threshold = 80 mg. per kilo

Dog 29-108, female, weight 15.5 kilos

Experiment, *days*......	1	2	3	4	6	7	8	9	10	Rest period, 2 mos.	1	2	3	4	5	6
Urine albumen....	0	2+	Tr.	0	0	0	2+	Tr.	0		0	2+	+	0	0	
HgCl₂, *mg. per kg*...	2	0	0	0	0	3	0		0		3	0	0	0	0	
Hb. intraven., *mg. per kg*..........	0	80	100	100	90	0	80	100	90		0	0	0	0	0	
Hb. in urine.......	0	0	0	+	+	0	+	+	+		0	0	0	0	0	
Clinical condition..	N	N	N	N	N	V	N	N	N		N	N	N	N	N	*

N = normal, V = vomiting.

* Killed and autopsy at once.

nephrosis (compare Dog 29-303, Table 34). There are some calcium deposits in the necrotic hyaline material within the convoluted tubules. The epithelial injury within the tubules is extreme. The glomerular tufts are normal.

Table 32 shows a somewhat different experiment. This dog tolerated doses of 2.0 and 3.0 mg. mercuric chloride per kilo with but slight albuminuria. The observations on the renal threshold for hemoglobin concern Paper II. The dog was then rested from hemoglobin injections for 2 months during which time blood (100 to 200 cc.) was removed from the jugular vein about twice a week. This bleeding reduced the red cell hematocrit from the normal level of 40 per cent to levels between 20 and 30 per cent. This moderate anemia was main-

tained with the expectation that the iron containing pigment in the tubular epithelium might be removed. This pigment did decrease markedly but small amounts were present at time of autopsy. After this 2 month period of anemia the dog was again tested with 3 mg. mercuric chloride per kilo and gave a response practically identical with that noted prior to the anemia interval. The tubular epithelium was as before more tolerant to mercury than the control (Dog 30-279, Table 33). The preceding period of injury and repair may be a factor

TABLE 33

Control Experiments—No Hemoglobin Injections
Dog 29-114, female, weight 16 kilos

Experiment, *days*.	1	2	3	4	5	7	8	9	10
Urine albumen................	0	4+	+	+	Tr.	Tr.		4+	
HgCl₂, *mg. per kg*..............	1	0	0	0	0	1.5 D	0 D	0	
Clinical condition.............	V	S	N	N	N	V	V	S	*

Dog 30-279, male, weight 28 kilos

Experiment, *days*.....................	1	2	3	4	7	8	9	10	12
Urine albumen................	0	0	Tr.	0	0	4+	4+		
HgCl₂, *mg. per kg*..............	0	1	0	0	2	0 D	0	0	
Clinical condition.............	N	N	N	N	V	V	S	S	**

N = normal, V = vomiting, D = diarrhoea, S = intoxicated.
* Death and autopsy at once.
** Died at night, autopsy next morning.

in this tolerance. Compare with a similar experiment (Paper II, Table 24, Dog 29-297) in which the anemia period was longer and the pigment deposits completely exhausted. This dog 29-297 then was somewhat less tolerant to subsequent injections of mercury.

Dog 29-108, Table 32. Dog was given gas and killed 6 days after the 3 mg. dose of mercuric chloride. Autopsy was performed at once. The gross findings are normal. Histological sections show in general normal renal tissues which would differ only slightly from that of normal stock dogs. There is no evidence of injury and repair of the convoluted tubular epithelium so that the injury must have been slight and completely repaired during the 6 day interval between the

mercury injection and the death of the dog. Pigment is present in small a[mounts?]
as very fine grains in the epithelium of the tubules in the renal cortex.
glomerular tufts are normal. The collecting and other tubules show occa[sional]
hyaline casts.

A control experiment for Dog 29-108, Table 32, was done at the same [time]
under identical conditions. Dog 30-279, Table 33. Died 5 days after the se[cond]
dose (2 mg. per kilo) of mercuric chloride. The dog died at night and autopsy [was]
done the next morning. The findings are of no significance except for the kid[neys]
which in gross were normal. Histological sections show the usual picture of [mercuric]
chloride nephrosis with extensive necrosis of tubular epithelium as noted bel[ow.]
Dog 29-303, Table 34. There is a moderate amount of postmortem autolysis.

TABLE 34

Resistance of Renal Epithelium to Mercury Poisoning Increased by Frequent
Hemoglobin Injections

65 hemoglobin injections given before this experiment
Dog 29-269, weight 15 kilos

Experiment, days	1	2	3	4	5
Urine albumen	Tr.	+	Tr.	Tr.	Tr.
HgCl₂, mg. per kg.	2.0	0	0	0	*
Clinical condition	N	N	N	N	N

Dog 29-303, weight 8.9 kilos (control experiment, no hemoglobin injections)

Experiment, days	1	2	3	4	5
Urine albumen	0	0	4+	4+	—
HgCl₂, mg. per kg.	0	1.5	0	0	0
Clinical condition	N	V	S	S	S*

N = normal, V = vomiting, S = intoxicated.
* Killed and autopsy at once.

Table 34 illustrates well the tolerance to mercuric chloride which is
observed in dogs given frequent intravenous injections of hemoglobin.
Dog 29-269 had received 65 injections in the weeks preceding the in-
jection of mercuric chloride. This dog tolerated a 2.0 mg. dose of
mercuric chloride per kilo with practically no evidence of renal injury
and no clinical disturbance. The control dog 29-303 was fatally
injured by a smaller dose, 1.5 mg. mercuric chloride per kilo. The
histological sections are in striking contrast, Dog 29-269 showing only
slight tubular injury and Dog 29-303, the control, shows an extreme

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nephrosis and destruction of tubular epithelium—see descript
under autopsy.

Dog 29-269, Table 34. Autopsy done at once after dog was given gas a
killed. The organs in gross present nothing of interest. The kidneys appe
normal except for some pigmentation. *Histological sections* in general are norma
as far as the glomeruli and tubular epithelium are concerned. The tubula
epithelium is pigmented and contains fine yellow granules noted in almost every
tubule in the cortex. The epithelium of the collecting tubules shows no pigment
but a few casts are noted. A few convoluted tubules show hyaline masses, evi-
dence of a recent necrosis and injury which is being repaired by the epithelium.
The amount of injury done the tubules by the dose of mercuric chloride 5 days
previously is certainly slight and has been practically completely repaired. Com-
pare this with the control dog 29-303 done at the same time with a smaller dose of
mercuric chloride and lethal result in 5 days.

Dog 29-303, Table 34. This dog was prostrated and obviously close to death.
He was given ether and killed. Autopsy was done at once. The kidneys are
practically normal in gross. *Histological sections* present the typical picture of a
bichloride nephrosis. The majority of the convoluted tubules show total hyaline
necrosis of their lining epithelium, the whole tubule being filled with a pink hyaline
mass. Some tubules show some residual epithelium and attempts at repair in
progress. The glomerular tufts appear normal. The collecting tubules show
casts but no cell injury. There are no deposits of calcium.

Table 35 illustrates again the tolerance to mercury which is the
rule in dogs which have been given frequent doses of hemoglobin in
the weeks preceding the mercury injection. The dog 29-250 toler-
ated the dose of 2 mg. mercuric chloride per kilo with trivial disturb-
ance. There was only a trace of albumen in the urine and the autopsy
4 days later shows minimal changes in the kidney tissues.

Dog 29-250, Table 35. Dog found dead 2 hours after injection of a rather large
dose of mercuric chloride—a death in shock resembling peptone shock. This is
4 days following a standard dose of 2 mg. mercuric chloride per kilo and the histo-
logical picture relates to this first injection. The autopsy was done at once and
presents the usual changes noted after death from shock. The kidneys were
normal in gross except for intense congestion. *Histological sections* show little
change from normal except congestion and a granular swollen epithelium in the
convoluted tubules which may be due in part to the shock and tissue absorption
of fluid. The glomerular tufts are normal except for congestion. Pigment is
abundant in the epithelium of the convoluted tubules and appears as fine grains of
yellow pigment obviously related to the frequent hemoglobin injections. Some
mitotic figures are seen in the epithelial cells of some convoluted tubules and this

may signify a reparative process. At any rate the renal tissues are practically normal and the repair from the mercuric chloride injury is complete.

Table 36 presents an atypical experiment in which the evidence is conflicting. The dog had been under observation for many months

TABLE 35

Resistance of Renal Epithelium to Mercury Poisoning Increased by Frequent Hemoglobin Injections

40 injections of sheep hemoglobin and 46 injections of dog hemoglobin, given before this experiment

Dog 29-250, weight 25 kilos

Experiment, days	1	2	4	5
Urine albumen				
HgCl₂, mg. per kg	0	0		
Clinical condition	2	0	Tr.	Tr.
	N	N	0	3
			N	*

N = normal.
* Death from shock of injection, autopsy at once.

TABLE 36

Questionable Effect of Few Injections of Hemoglobin as Influencing Resistance of Renal Epithelium to Mercury Poisoning

Lowest normal or depression renal threshold = 84 mg. per kilo

Dog 29-258, male, weight 26 kilos

Experiment, days	1	2	3	4	5	6
Urine albumen	0	4+	2+	2+	2+	
HgCl₂, mg. per kg	2	0	0	0	0	
Hb. intraven., mg. per kg	0	90	90	0	0	0
Hb. in urine	0	+	0	0	0	0
Clinical condition	N	V	V	D	S	*

N = normal, V = vomiting, D = diarrhoea, S = intoxicated.
* Death and autopsy at once.

and in the first part of the period he was given a good many hemoglobin injections. Then came a rest period of 1 month followed by 8 injections of hemoglobin. Then came a rest period of 2 months followed by 11 injections of hemoglobin which immediately preceded this experiment in Table 36. A dose of 2 mg. mercuric chloride per kg. caused a fatal renal injury with death on the 6th day. Either these

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hemoglobin injections gave this dog no protection or he was unusually susceptible to mercury. The hemoglobin injections immediately preceding this experiment are much less in number than was true for the other dogs which gave evidence of tolerance for mercuric chloride.

Dog 29-258, Table 36. Death took place on the 6th day after the mercury injection and the autopsy was done at once. The anatomical findings other than those in the kidneys do not concern these experiments. The kidneys were normal in gross. *Histological sections* show the familiar picture of a bichloride nephrosis. The epithelial destruction is complete in many tubules and severe in others where much fat is noted. The evidences of epithelial regeneration are conspicuous. Pigment is found in small amounts and in fine grains in a few of the tubules. Some of the damaged epithelium contains fine grains of yellow pigment. The glomerular tufts are normal. Casts are everywhere numerous. There are no deposits of calcium.

DISCUSSION

In normal stock dogs we find that mercuric chloride administered intravenously as described will cause a fatal renal injury in small doses of 1.5 to 2.0 mg. per kilo. Some of these observations are given in Tables 33 and 34. This minimum lethal dose is somewhat below that recorded by Sansum (3) and Haskell, Hamilton and Henderson (1). When normal dogs have been given frequently repeated (40 to 80 times) intravenous injections of dog hemoglobin in amount close to the renal threshold values, we note an accumulation of yellow granular pigment in the epithelial cells of the convoluted tubules (Paper IV). Coincident with this the dog becomes somewhat resistant to mercuric chloride and will tolerate the normal minimal lethal dose with little if any evidence of injury of the tubular epithelium—in fact some dogs will tolerate 3 to 4 mg. per kilo with little evidence of renal injury. Large doses of mercuric chloride uniformly cause fatal renal injury. We may choose to believe that this pigment material neutralizes in part the mercuric salt and renders it much less toxic to the tubular epithelium. Or we may say that this epithelium is so stuffed with pigment material that it does not take up the mercury as promptly or completely and therefore the epithelium escapes severe injury. Whatever our explanation this tubular epithelium reacts differently to minimal lethal doses of mercuric chloride.

This is an illustration of the fact that slight changes in cells may

modify considerably their reaction to poisons or stimuli of various sorts. These changes are within physiological limits and we cannot say that the tubular epithelium is actually injured by this deposit of pigment and related materials because renal function goes on quite normally. Moreover this pigment will disappear during resting periods especially if anemia due to bleeding is produced. Evidently this whole process of pigment conservation is within the normal range of the physiological capacities of the tubular epithelium.

This reaction is of the order of that observed by MacNider (2) who noted that after a sublethal dose of uranium the dog renal tubules repaired this injury with epithelium that in places was somewhat different from normal. Subsequently he observed that these kidneys are more tolerant to uranium poisoning.

SUMMARY

Frequent injections of superthreshold amounts of dog hemoglobin will cause deposits of pigment material in the renal tubular epithelium. When this has happened this dog will survive minimal lethal doses of mercuric chloride with little evidence of renal injury. In fact some dogs will tolerate twice the minimal lethal dose without severe reaction.

There is no evidence that continued injections of dog hemoglobin in these amounts will cause any injury or functional disability of the kidney. Rest periods will effect a disappearance of this pigment in the renal tubules.

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IV. HEMOGLOBIN INJECTIONS AND CONSERVATION OF PIGMENT BY KIDNEY, LIVER AND SPLEEN

THE INFLUENCE OF DIET AND BLEEDING

By WILLIAM V. NEWMAN, M.D., AND GEORGE H. WHIPPLE, M.D.

(From the Department of Pathology, The University of Rochester School of Medicine and Dentistry, Rochester, N. Y.)

PLATE 25

Our interest in the conservation of hemoglobin in anemia has been touched upon in the first paper of this series. Having demonstrated that injected hemoglobin was completely conserved in experimental anemia (9), we wished to understand the mechanism of this reaction and its related internal body metabolism. That the liver and spleen played significant rôles in this conservation was to be anticipated but the importance of the kidney, unexpected as it was, comes out clearly in the experiments tabulated below. We now feel that it can be stated with confidence that the kidney must rate in importance close to the liver and spleen in relation to hemoglobin conservation under certain conditions. This applies particularly to various types of hemoglobinemia which approach the minimal renal threshold levels as established in Paper I.

When a considerable number of daily hemoglobin intravenous injections are given close to or above the minimal renal threshold we find abundant deposits of iron staining (hemosiderin) pigment in the kidney, liver and spleen. From the amount of such pigment in the liver and spleen however, one cannot estimate even approximately the total amount of hemoglobin injected. In the kidney if the daily hemoglobin injections are continued at a level below the minimum or depression or glomerular threshold (Paper I) we find no hemosiderin in the epithelium of the convoluted tubules even though 48 to 54 gm. hemoglobin be given over a considerable period (3 months) (Dogs 29-110 and 30-84, Table 41).

In the experiments where the hemoglobin injections are close to or just above the depression renal threshold level, we find hemosiderin in the epithelium of the convoluted tubules and this deposit increases as the superthreshold injections are multiplied. Up to a certain point the epithelium of the renal tubules continues to absorb pigment from the lumen but they have a saturation point beyond which they seem unable to take up more hemoglobin. It would seem probable that as this renal epithelium takes up hemoglobin and digests it to hemosiderin or other related pigments, it might store other digestion products coming from the ingested hemoglobin. This would fit in with the well known fact that kidney feeding in anemia favors new hemoglobin construction and indicates that the kidney tissue is rich in the "building stones" which the body utilizes to fabricate new hemoglobin in anemia.

This pigment material in kidney, liver and spleen will slowly disappear during non-injection or resting periods. Bleeding and anemia accelerate this clearing out of pigment material from these organs and we may safely conclude that some of this material will be conserved and rebuilt into much needed new hemoglobin and red cells.

Pigment deposits in liver, spleen and kidney have been observed by numberless workers but usually as a result of some procedure which destroyed the red cells within the circulation. Muir and McNee (3) studied anemia produced in animals by hemolytic sera and observed hemosiderin in liver, spleen and kidneys. Boycott and Price-Jones (1) investigated trypanosome anemia. Muir and Dunn (4) also used hemolytic sera to cause anemia and studied the iron containing pigment in the kidney, liver and spleen. We felt that a study of injected hemoglobin presented some advantages as we knew just how much hemoglobin was set free in the circulation, also that only hemoglobin and not red cell fragments were added to the circulation.

Methods

Healthy adult dogs were used in all experiments. Some of the material tabulated below was derived from the dogs whose histories are given in the first three papers of this series. The preparation of dog hemoglobin is described in Paper I. Sheep hemoglobin was prepared in the same way. Goose hemoglobin was prepared as follows: The blood was drawn from a neck vein, defibrinated with glass beads and filtered through gauze. The red cells were washed twice with normal saline and thrown down by centrifugalization. To each 1 cc. of packed red cells

were added 2 cc. of distilled water, the cells laked by shaking, the residue removed by the centrifuge and the solution finally filtered through gauze. A sample was taken for the determination of the amount of hemoglobin (5). The muscle hemoglobin was prepared after the method as described by Whipple (6).

At the end of the experiment the dog was bled and perfused to remove the circulating red cells as described in Paper V. This applies to all dogs in which iron analyses are recorded. Without this precaution the iron analyses have little significance as the red blood cells in the capillaries may contain more iron than the entire parenchyma cells of the organ.

Because of the perfusion (Paper V) the organs are very pale at autopsy. The kidneys are a pale buff color. The liver is a pale yellowish brown and the spleen is a pinkish red color. The lymph glands usually show a pink tint. The tables record the findings in the mesenteric lymph glands. The gastrointestinal tract is pale and white. Sections of all tissues were taken in Zenker's fixative, cut in paraffin, stained with hematoxylin and eosin and with Mallory's iron stain (ferrocyanide). Bone marrow was examined as a routine but these sections are not included in our tables as the marrow runs in parallel with the spleen.

The amounts of iron staining pigment are recorded in the tables as numbers—arbitrary units beginning at 1 which is but little more than a trace, running up to 5 which is the maximal amount observed. These figures were reviewed independently by the writers but at best represent a rough estimate. Comparison with accurate iron analyses shows no parallelism between the iron values and the iron staining pigment as estimated in Table 41. This was to be expected as it is realized that the amount of masked or combined iron which gives no iron staining reaction may be very large.

The complete iron analyses of this series of dogs is given in the last paper of this group (Paper V) but in certain tables given below the interesting iron figures are included for the sake of ready comparison with the arbitrary figures indicating the amount of iron staining pigment in the kidney, liver and spleen.

Table 41 shows two significant findings. (1) Even a large total dosage of dog hemoglobin given in daily amounts well *below the minimal renal threshold* will cause no deposit of iron staining pigment in the convoluted tubules (Dogs 30-84 and 29-110). By contrast a few doses of hemoglobin above the minimal renal threshold will effect an easily recognizable deposit of iron staining pigment in the convoluted tubules (Figs. 1 and 2). (2) Muscle hemoglobin has a very low renal threshold but the epithelium of the convoluted tubules does not take up an iron staining pigment as a result of repeated superthreshold doses of muscle hemoglobin. In this respect muscle hemoglobin differs from blood hemoglobin whether obtained from red cells of the dog, sheep or goose.

Table 41 gives the essential features of each experiment as tabulated but the following notes give some details which would be of value to other workers in this field who might wish to compare carefully these experiments with some of their own. These notes follow the order observed in Table 41.

Dog 29-252 (Table 41) was given very large doses of dog hemoglobin for a period of only 7 days. Each dose caused a conspicuous hemoglobinuria. The histological material showed only a moderate amount of iron staining pigment in the liver,

TABLE 41

Hemoglobin Injection, Iron Staining Pigment and Iron Analyses

Dog No.	Wt.	Hemoglobin injected				Pigment giving iron stain				Iron = mg. per 100 gm. of tissue			
		Type	Mg. per kg. daily	Total	Days inj.	Kidney	Liver	Spleen	Lymph gland	Kidney	Liver	Spleen	Lymph gland
	kg.			gm.									
29-252	7.5	Dog	625	31	7	3	1	2	1	44.8	37.5	177.1	13.8
30-135	17.8	Dog	125	56	25	4	4	5	3	17.4	36.8	122.7	22.6
29-213	15.2	Dog	73	32	29	0	1	1	1	11.0	27.2	40.2	15.4
30-84	16.5	Dog	35	48	85	0	3	3	1	6.2	45.3	101.9	
29-110	17.0	Dog	35	54	95	0	1	1	3				
29-219	14.7	Sheep	147	32	15	1	1	2	2	32.7	30.7	144.6	30.6
29-228	29.5	Sheep	85	42	17	2	1	2	2	25.7	34.3	58.7	
30-108	5.3	Goose	124	10	15	3	2	5	—	35.2	20.9	111.8	
30-231	12.7	Goose	80	15	15	0	2	2	2	17.4	21.1	43.8	10.0
30-149	17.5	Muscle	17	3.6	15	0*	2	4	3	14.1	31.4	107.6	
30-228	14.0	Muscle	12	2.5	16	0	3	4	2	9.1	26.6	143.5	

* Endothelial cells contain some iron staining pigment.

spleen and lymph gland. The kidney showed abundant iron staining pigment in the convoluted tubules. Hemoglobin casts were numerous and some contained pigment which gave a positive iron stain.

Dog 30-135 (Table 41) was given graduated doses of dog blood hemoglobin. 110 mg. per kilo of body weight were injected for 5 days and the dose increased to 150 mg. for 5 days. Subsequent injections were stepped up 10 mg. per kilo and given for 5 days each until hemoglobinuria was obvious. A total of 56 gm. was injected over a period of 25 days. Histological examination of the kidney showed a very large amount of finely granular pigment in the convoluted tubular

epithelium which was hard to see and suggested recent deposits. Fom this picture we assumed that much of this pigment was deposited in the tubular epithelium during the last 5 days of injection when hemoglobinuria was pronounced. The liver contained a large amount of iron staining pigment.

Dog 29-213 (Table 41) was injected with hemoglobin doses which evidently were very close to the minimal renal threshold level. The kidney showed no iron staining pigment in the convoluted tubular epithelium. The liver, spleen and lymph gland all showed low values in spite of rather large total hemoglobin dosage. We have no explanation except the possibility that this dog was a bit anemic to start with and utilized this material promptly instead of storing it as usual.

Dog 30-84 (Table 41) received hemoglobin doses which were certainly well below the minimal renal or glomerular threshold and there are no iron staining pigments in the convoluted tubular epithelium although the total dosage of 48 gm. is high and the injections continued over a period of 85 days. Liver and spleen show abundant pigment deposits.

Dog 29-110 (Table 41) also received daily doses of dog hemoglobin which were definitely below the minimal renal threshold. No iron staining pigment appears in the epithelium of the convoluted tubules in the kidney. The total dosage was even larger than the preceding experiment (Dog 30-84) and continued over a period of 95 days.

Dog 29-219 (Table 41) was given superthreshold doses of sheep hemoglobin which caused a daily hemoglobinuria. This was continued for 15 days and as was to be expected, the kidney showed iron staining pigment in its convoluted tubules.

Dog 29-228 (Table 41) was given rather small amounts of sheep hemoglobin daily which however in every instance caused a readily observed hemoglobinuria. The kidney showed the usual iron staining deposits in the convoluted tubules. It is interesting to note that this dog received less sheep hemoglobin than was given in the preceding experiment but had more iron staining pigment in the kidney. The iron analysis figures are in harmony with the injection totals rather than with the iron staining pigment.

Dog 30-108 (Table 41) was given definite superthreshold doses of goose hemoglobin which caused distinct hemoglobinuria after each injection. The kidneys showed large amounts of iron staining pigment in the epithelium of the convoluted tubules—also in the liver and spleen.

Dog 30-231 (Table 41) was given doses of goose hemoglobin which evidently were very close to the glomerular threshold. Possibly a little hemoglobin may have seeped through the glomeruli as the iron analysis shows a slight increase in the kidney. There was no deposit of pigment in the epithelium of the convoluted tubules. At no time during the experiment was hemoglobinuria noted.

Dog 30-149 (Table 41) was given doses of dog muscle hemoglobin which were definitely superthreshold and hemoglobin appeared daily in the urine after each injection. The very low renal threshold for muscle hemoglobin is one of the peculiar features of this interesting substance. In spite of obvious hemoglobinuria we record no deposits of iron staining pigment in the epithelium of the convoluted

tubules. Here again this muscle hemoglobin differs from blood hemoglobin as to its physiological behaviour in the dog. There is abundant iron staining pigment in the liver, spleen and lymph gland. A little iron staining pigment is noted in the endothelium of the renal cortex between the tubules.

A yellow granular pigment negative for iron is noted in these dogs injected with muscle hemoglobin and is abundant in the convoluted tubular epithelium and in the hepatic epithelium (see Table 45). The Kupffer cells of the liver contain some iron staining pigment.

Dog 30-228 (Table 41) was given smaller doses of muscle hemoglobin which caused hemoglobinuria on some occasions. The autopsy findings were in all respects similar to the preceding experiment—Dog 30-149.

Figs. 1 and 2 show the characteristic iron staining deposits within the epithelium of the convoluted tubules of the kidney. The cell nuclei are very faintly stained with carmine and the blue staining pigment stands out conspicuously by contrast. Fig. 1 is a low power microphotograph of the kidney cortex and Fig. 2 is a higher power picture of a group of tubules in the same kidney.

Table 42 gives the pigment findings in some of the dogs used in Papers I to III. The histories of these dogs will be found in detail in these preceding papers. As no perfusions were done it was obviously a waste of energy to perform iron analyses. Very brief histories of these dogs in the order found in Table 42 are noted. Dog 28-233 (Table 42) received both subthreshold and superthreshold hemoglobin injections over a considerable period. The iron staining pigment was very abundant in the epithelium of the convoluted tubules of the kidney. The same pigment was abundant in the endothelial cells in the kidney cortex between the tubules.

Dog 29-198 (Table 42) was given a long series of dog hemoglobin injections— see Paper II, Table 22. There were repeated sublethal injections of mercuric chloride. He was given superthreshold and subthreshold hemoglobin injections. Much iron staining pigment was found in the epithelium of the convoluted tubules in the kidney cortex.

Dog 29-26 (Table 42) was given a long series of dog hemoglobin injections, many of which were superthreshold in amount with consequent hemoglobinuria.

Dog 29-334 (Table 42) was given a short series of hemoglobin injections close to the minimum renal threshold. There was slight hemoglobinuria observed at times. Repeated sublethal doses of mercuric chloride were given.

Dog 30-32 (Table 42) received a series of hemoglobin injections which were very close to the minimal renal threshold. On several occasions there was noted slight

hemoglobinuria—see Paper II, Table 21. Mercuric chloride also was given. Very little iron staining pigment was found in the kidney, probably due to the few superthreshold hemoglobin injections.

Dog 30-38 (Table 42) received a long series of hemoglobin injections—see Table 31, Paper III. Hemoglobinuria was noted frequently and mercuric chloride was given. At autopsy abundant iron staining pigment was found in the usual locations in kidney, liver and spleen.

Dog 29-250 (Table 42) received both sheep and dog hemoglobin injections, some of which were superthreshold and caused hemoglobinuria. Mercuric chloride was given—refer to Paper III, Table 35.

Dog 29-304 (Table 42) received a few doses of sheep hemoglobin followed by

TABLE 42

Hemoglobin Injection and Iron Staining Pigment

Dog No.	Wt.	Hemoglobin injected				Pigment giving iron stain			
		Type	Mg. per kg. daily	Total	Days inj.	Kidney	Liver	Spleen	Lymph gland
	kg.			*gm.*					
28-233	19.5	Dog	130	112	120	4	2	3	1
29-198	18.6	Dog	120	130	210	4	2	3	1
29-26	15.0	Dog	400	160	175	4	1	3	—
29-334		Dog	110	55	35	1	1	1	—
30-32	19.0	Dog	85	76	44	1	3	5	5
30-38	19.0	Dog	110	90	100	3	5	5	—
29-250	25.0	Dog	170	125	180	2	1	2	—
		Sheep	64						
29-304	20.6	Dog	97	59.0	60	3	1	—	—
		Sheep	39	5.7	10				
30-107	6.0	Goose	80	9	21	2	1	2	1

many of dog hemoglobin, some of which were above the minimum renal threshold. The iron containing pigment was abundant in the kidney.

Dog 30-107 (Table 42) received a series of injections of goose hemoglobin. The renal threshold was exceeded with a daily record of hemoglobinuria. Autopsy showed the usual deposit of iron staining pigment in the convoluted tubular epithelium of the kidney.

The diet experiments in Table 43 should be compared with the anemia experiments in Table 44. The data are regrettably meagre but merit some discussion and indicate the nature of future experiments which would supply much needed information. Our stock dogs maintain a high hemoglobin level which in our laboratory is 120 to

rapidly to an anemia level of 50 to 60 per cent hemoglobin and maintained at this level for the total duration of the experiment.

Dog 30-271 (Table 44) showed a moderate amount of golden yellow pigment negative for iron in the convoluted tubular epithelium and endothelial cells of the kidney. A few areas of scarring were noted in which some phagocytes were present that contained iron staining pigment. The liver epithelium and Kupffer cells contained a moderate amount of yellow pigment (Table 45) which was negative for iron except for a trace in the Kupffer cells. Iron staining pigment was present in small quantity in the spleen. The lymph gland showed a moderate amount of pigment, occasional grains of which took the iron stain.

Dog 30-257 (Table 44) showed moderate amounts of golden yellow pigment

TABLE 44

Hemorrhage and Anemia as Influencing Iron Staining Pigment and Iron Analyses

Dog No.	Wt.	Anemia level Hb.	Anemia dura-tion	Pigment giving iron stain				Iron = mg. per 100 gm. tissue			
				Kidney	Liver	Spleen	Lymph gland	Kidney	Liver	Spleen	Lymph gland
	kg.	*per cent*	*mos.*								
30-271	24	50–60	1	0	1	1	1	1.4	5.0	17.5	
30-257	17	50–60	1.5	0	0	1	0	2.9	4.0	6.6	
21-23	14	40–50	64	0	0	1	—				
20-104	8	40–50	60	0	0	0	—				
25-24	10	40–50	22	0	0	0	—				
29-288	—*	95–100	3	0	0	1	1	3.0	13.8	15.3	9.4
29-8	—**	95–100	1±	0	1	1	2	3.9	11.4	52.0	8.3

* Spontaneous hematuria.
** Closed sterile bile fistula for 9 months.

negative for iron in the kidney epithelium, liver epithelium and Kupffer cells and a small amount in the lymph gland (Table 45). The spleen contained a small amount of pigment which gave a positive iron stain.

Dog 21-23 (Table 44) was kept at an anemia level of 40 to 50 per cent hemoglobin for 64 months. The diet for the last 9 weeks consisted of standard anemia bread and salmon. Fourteen injections of dog muscle hemoglobin were given 3 weeks before death. Four days before death 2 gm. of dog blood hemoglobin were injected intravenously. The dog was killed by accident related to a hemoglobin injection. The convoluted tubular epithelium and endothelial cells of the kidney contained heavy deposits of golden yellow pigment and traces in the epithelium of the collecting tubules. This pigment was negative for iron and hemofuscin. A moderate amount of this same type of pigment was found in the liver epithelium and Kupffer cells (see Table 45). The spleen contained a trace of iron staining pigment.

Dog 20-104 (Table 44) was maintained at an anemia level of 40 to 50 per cent hemoglobin for 60 months. His diet for the 10 weeks preceding death was made up largely of standard anemia bread to which during some weeks were added pig muscle, beef muscle, pig heart and pig liver. A large amount of golden yellow pigment was found in the kidney epithelium, a large amount of this pigment was present in the liver epithelium and Kupffer cells, and a small amount in the spleen. None of this pigment took the iron stain except for a few tiny granules in the trabeculae of the spleen (Table 45).

Dog 24-24 (Table 44) was kept at an anemia level of 40 to 50 per cent hemoglobin for 22 months. Death was accidental. His diet for the 10 weeks preceding death consisted of standard anemia bread. During the last 3 weeks of life, liver and ferric chloride (Fe equivalent 300 mg.) were added to the diet. This diet is of maximal potency for the regeneration of new hemoglobin under these conditions. The kidneys contained large deposits of golden yellow pigment in the epithelium of the convoluted tubules and traces in the collecting tubules. In the liver large quantities of this pigment were present in the liver epithelium and Kupffer cells. The spleen contained a small amount of golden yellow pigment. None of the pigment found in the tissues of this dog gave a positive iron stain.

Dog 29-288 (Table 44) had spontaneous hematuria for 3 months. The amount of bleeding was not great and the anemia was only of moderate grade. He was perfused and autopsied. The cause of the hematuria could not be determined but we may believe that some small vessel in the urinary tract was contributing this blood to the urine. The kidneys and liver contained no pigment. Phagocytic cells in the spleen and lymph gland contained a small amount of iron staining pigment. We note that even this low grade of anemia (2/3 normal hemoglobin) reduces the iron staining pigment and lowers the iron content below the normal controls (Table 43).

Dog 29-8 (Table 44) had a sterile closed bile fistula for 9 months. She became obstructed and jaundice developed. She was killed under ether anaesthesia with the usual perfusion. The kidneys showed no pigment. The liver and spleen contained a small amount of iron staining pigment; the lymph gland a moderate amount of this type of pigment.

This bile fistula animal (Table 44, Dog 29-8) presented only a slight degree of anemia—about 2/3 normal hemoglobin, but the spleen shows evidence of a high iron content although the iron staining pigment is not conspicuous. We may assume that the obstructed fistula was responsible for this blood destruction and low grade anemia.

Table 45 deals with an entirely *different pigment*—one which gives no stains for iron and no reaction for hemofuscin. The tissues may be rich in this pigment yet poor in iron according to iron analysis (Dogs 30-271, 30-257 and 30-270). One is tempted to designate it as a lipochrome but this type of pigment is not commonly found in the

kidney. It is somewhat more highly refractile than the usual hemosiderin pigment. It interested us particularly as it seems to be more abundant in the kidney suggesting the possibility that the kidney was conserving this pigment just as it does hemoglobin and its split products under certain conditions.

TABLE 45
Pigment Deposits Giving a Negative Iron Stain

Dog No.	Diet daily	Refer to Table	Anemia		Yellow granular pigment			Iron = mg. per 100 gm. tissue		
			Hb. (per cent)	Duration (mos.)	Kidney	Liver	Spleen	Kidney	Liver	Spleen
30-271	Anemia / Bread and milk	44	50–60	1	2	2	1	1.4	5.0	17.5
30-257	Anemia / Bread and milk	44	50–60	1.5	2	2	0	2.9	4.0	6.6
21-23	Anemia / Bread	44	40–50	64	5	2	0			
25-24	Fe (300 mg.) / Bread + salmon	44	40–50	22	4	4	1			
20-104	Anemia / Bread	44	40–50	60	4	3	0			
30-103	Liver (300–400 gm.) / Bread + meat	43	120–150	—	3	0	0	5.4	30.3	46.1
30-270	Carrot + mush / Kennel	43	120–150		2	0		5.0	42.2	53.6
30-228	Kennel	41	120–150	*	4	3	0	9.1	26.6	143.5
30-149	Kennel	41	120–150	*	3	4	0	14.1	31.4	107.6

*Muscle hemoglobin injections intravenously.

In long continued anemia in dogs we have observed a pigmentation of the gastrointestinal muscular coats which take on a buff color in many animals. This pigment however is soluble in alcohol. We have suspected liver feeding as being at least in part responsible for this peculiar pigment deposit.

The pigment observed in Table 45 obviously is not soluble in alcohol otherwise it would be removed in the routine preparation of histological sections. We cannot say what factors may be responsible for this non-iron staining pigment and it is possible that some of it may be

hematoidin. We suspect three factors mentioned as follows: (1) Muscle hemoglobin injections were given the last two dogs in Table 45 (Dogs 30-228 and 30-149). This suggests the possibility of hematoidin or of some other pigment associated with the muscle hemoglobin. (2) Liver or cod liver oil feeding in Dogs 21-23, 25-24, 20-104, and 30-103 may be responsible. However not all dogs given such diets will show this pigment. The standard anemia bread used in many of these experiments (Table 45) contains much cod liver oil and also canned salmon. (3) Salmon muscle feeding may be responsible as this food contains a pigment but not all dogs given this diet may show this pigment deposit. Obviously the source of this pigment deposit (Table 45) is not clear and future work must determine its make up and causative factors.

DISCUSSION

We have reviewed experiments in the first paper of this series to show that the *initial renal threshold* for dog hemoglobin in dogs will be depressed by daily injections of hemoglobin in amounts close to the threshold level. This minimal renal threshold for hemoglobin is relatively constant if the daily hemoglobin injections are continued and averages 45 per cent below the initial level. During the time when the renal threshold is falling toward the minimum level the epithelium of the convoluted tubules of the kidney is taking up iron containing pigment presumably related to the injected hemoglobin. When this tubular epithelium can no longer take up more hemoglobin we believe the minimum renal threshold is attained. This corresponds closely to the true glomerular threshold as described above (Paper I).

Furthermore we have shown in Paper II that moderate injury of tubular epithelium by mercuric chloride does not modify this minimum renal threshold level. It is difficult to believe in the face of this evidence that the epithelium of the convoluted tubules plays an active part in the escape of hemoglobin from the circulating blood. We prefer to think of the glomerular tuft as fixing the true renal threshold. This level may be modified for bladder urine by absorption of hemoglobin from the tubular lumen.

The observations tabulated in this paper fit in quite satisfactorily with this thesis of hemoglobin elimination by way of the glomerular

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tuft.   The amount of iron staining pigment within the epithelium of the convoluted tubules of the kidney increases with the number of hemoglobin injections which exceed the renal threshold levels.   When a large number of hemoglobin injections are given in amounts below the minimum renal threshold (Dogs 30-84 and 29-110, Table 41) no iron staining pigment appears in the tubular epithelium although the liver and spleen show the usual heavy pigmentation.

Muscle hemoglobin behaves differently when introduced into the blood stream and has a very low renal threshold—less than 10 per cent of dog blood hemoglobin.   In spite of the fact that the muscle hemoglobin escapes so readily in the urine, the tubular epithelium of the kidney does not accumulate any iron staining pigment.   These dogs do present a yellow pigment negative for iron included in the epithelium of the renal tubules.   At the present time we have no suggestions as to the make up of this pigment.

Muscle hemoglobin is a very interesting and probably important substance so that everything related to it has an appeal for the physiologist.   It is much like blood hemoglobin when we examine it by means of the spectrophotometer and when introduced into the blood stream bile pigment is promptly split off (8) just as in the case of blood hemoglobin.   Yet it has precipitin reactions which differentiate it from blood hemoglobin (2).   This last observation adds one other feature in which muscle hemoglobin differs from blood hemoglobin—as it passes through the lumina of the renal tubules there is no deposit of iron staining pigment within the tubular epithelium.

SUMMARY

When the minimal renal threshold for blood hemoglobin is exceeded there is observed a deposit of iron staining pigment in the epithelium of the renal convoluted tubules.   At a certain point this epithelium cannot take up more hemoglobin and this coincides with the minimal renal threshold level.

When the injections of blood hemoglobin are kept below the minimal renal threshold level we note a complete absence of iron staining pigment in the renal tubular epithelium.

Given a deposit of iron staining pigment in the tubular epitheliu[m] it will slowly disappear during rest periods with no hemoglobin inj[ections]. Anemia due to bleeding will accelerate this removal of pigme[nt] from the renal epithelium and this indicates a conservation of materia[l] by the kidney for use in construction of new hemoglobin.

Pigment giving a positive stain for iron will be found in the liver an[d] spleen when hemoglobin injections are given, regardless of the renal threshold. Removal of this pigment is accelerated by anemia due to bleeding and as a rule an anemia period of 2 months at a level of 1/3 normal (40 to 50 per cent hemoglobin) will render the spleen, liver and kidney free from iron staining pigment.

Pigment giving a positive iron stain is frequently observed in the mesenteric and lower retroperitoneal lymph glands. This is merely a drainage of pigment and phagocytes including pigment from some organ in which the pigment deposit was primary.

In stock dogs in this laboratory the hemoglobin level is quite high when the animals are in a perfectly normal state. The blood hemoglobin averages 120 to 150 per cent hemoglobin. In such dogs iron staining pigment in the spleen is a common finding and on occasion is observed in the liver.

To establish an accurate base line for the study of iron and iron staining pigment storage due to diet intake one must submit these dogs to a preliminary anemia period of at least 2 months.

Muscle hemoglobin has a very low renal threshold and escapes freely into the urine when given intravenously. Contrasting with blood hemoglobin this muscle hemoglobin under identical conditions does not cause the deposit of iron staining pigment within the epithelium of the renal tubules.

A pigment giving no iron staining reaction may be found in the epithelium of the convoluted tubules of the kidney. Whether this is due to dietary or other factors is uncertain but this indicates pigment conservation by the kidney.

Finally we would emphasize again the fact that the kidney is of considerable importance in the conservation of hemoglobin and hemoglobin split products which presumably are utilized to build up new hemoglobin.

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EXPLANATION OF PLATE 25

FIG. 1. Distribution of iron staining pigment in section of renal cortex.   × 107.
FIG. 2. Iron staining pigment in epithelium of tubules and in phagocytes.
× 529.

1
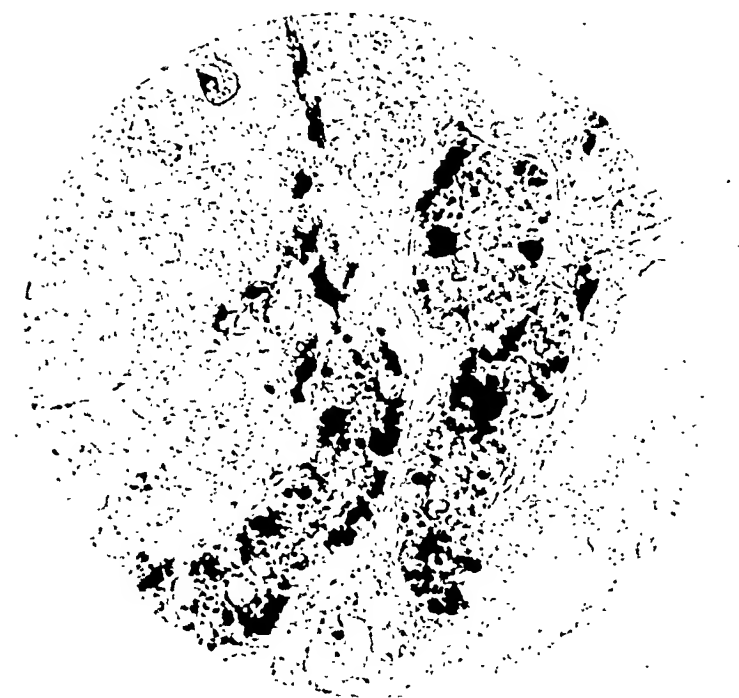
2

V. THE IRON CONTENT OF BLOOD FREE TISSUES AND VISCERA

VARIATIONS DUE TO DIET, ANEMIA AND HEMOGLOBIN INJECTIONS

BY ROBERT P. BOGNIARD, M.D., AND GEORGE H. WHIPPLE, M.D.

(From the Department of Pathology, The University of Rochester School of Medicine and Dentistry, Rochester, N. Y.)

When one studies the conservation of hemoglobin in the body, a knowledge of the storage of iron in various tissues is much to be desired. This information could not be obtained from the literature on this subject extensive as it is. It will be found that the distribution of iron corresponds roughly with the deposit of iron containing pigment (Paper IV) but there are many discrepancies which are obviously explained by iron deposits which give no iron stain.

When one reviews the published experiments of many investigators dealing with the iron content of various organs, it becomes apparent that two serious difficulties must be faced. Iron analyses are difficult[1] when applied to viscera and method errors may actually mask the true iron content. Analysis of viscera containing blood within the capillaries gives no accurate measure of the tissue iron as the contained blood may contain more iron than the tissue. We believe we have overcome both of these difficulties.

A glance at the tables below shows that the *kidney* as well as the spleen, bone marrow and liver is concerned with the conservation of iron after frequent hemoglobin injections. In Paper IV we have discussed the iron staining pigment found in the epithelium of the convoluted tubules of kidney. When the minimal renal threshold for hemoglobin is exceeded, we note a deposit of iron staining pigment in the tubular epithelium but there are no pigment deposits of this

nature if the hemoglobin injections are kept below the minimal renal threshold. The iron analyses of the kidneys are in harmony with these observations. With subthreshold hemoglobin dosage the kidney shows a normal or control iron content but following many super-threshold hemoglobin injections the iron content of the kidney may increase to 5 or 6 times the control level (Table 53).

Under a variety of dietary conditions we observe that the iron content of spleen, liver and bone marrow fluctuates widely but the kidney is relatively constant. We believe this is in part due to the fact that our dogs maintain an unusually high hemoglobin level (120 to 150 per cent) and presumably have a high storage of iron and iron staining pigment.

Anemia reduces the storage of iron staining pigment (Paper IV) as well as the tissue iron (Table 52) which indicates that this stored iron is used at least in part for the elaboration of new hemoglobin. To attain the most accurate base line in the *study of iron storage* due to diet or other factors we believe an initial anemia period of 2 months or more is highly desirable.

Methods

Healthy adult dogs were used in all experiments with the few exceptions specifically mentioned. These normal dogs presented a red cell hematocrit of 50 to 55 per cent which corresponds to 140 to 150 per cent hemoglobin. Some method procedures have been described in the other papers of this series. The pigment studies of these same dogs are given in Paper IV.

The body tissues and viscera were freed from red cells by a method previously described (4). In brief, the method is as follows: Under ether anaesthesia the jugular vein and carotid artery are canalized. A Locke's solution containing 5 per cent glucose flows by gravity into the jugular while simultaneously equal amounts of blood are removed from the carotid. By the use of graduated containers the inflow of Locke's solution and the outflow of blood is kept equivalent and there are no large fluctuations in the blood volume. Under such conditions with ether anaesthesia the heart continues to beat with its usual force until the end of this viviperfusion and the tissues are washed free of red cells as in no other way. If the bleeding is not too rapid the procedure occupies about 30 minutes and the red cell hematocrit falls from 50 to 55 per cent or normal to a fraction of 1 per cent which we consider representative of a successful type of perfusion. There is no gross edema of tissues to confuse the picture as is true even with an unsatisfactory gravity perfusion. At the end of the perfusion the heart beats begin to fail and at this time a little adrenalin given through

the jugular will raise the blood pressure for a time and facilitate the washing out of red cells within the viscera. When the heart stops the viscera are removed promptly, permitted to drain, placed in a towel moistened with Ringer's solution to prevent drying, then cut up and weighed for analysis. The ribs used for analysis while fresh are scraped free of all muscle and periosteum and no cartilage is included. Small bits of tissue are saved for histological study.

In a satisfactory experiment the tissues are bloodless in gross and show no red cells in histological section with the exception of the spleen and bone marrow. The lungs, gastrointestinal tract and pancreas are a pale grayish white. The heart and striated muscle present the usual color due to muscle hemoglobin which is not washed out. The spleen is small and a pale pinkish red. The liver is a deep buff or maple sugar color. The kidneys are a pale buff often modified by pigment of a brown color. The rib marrow appears as usual and is a pale pink color. The lymph glands are often a bit pigmented. The weights of the specimens taken for iron analysis depend upon the mass of tissue available. The weights run from a few grams in the case of lymph glands up to 80 to 90 gm. in the case of the liver.

Muscle hemoglobin solutions used in Table 53 were prepared by method previously described in detail (4).

The method for *iron analysis* is accurate and dependable but somewhat time consuming. It is described in detail in a recent publication by Bassett, Elden and McCann (1). The dry method of ashing by means of the electric furnace was found to be unsatisfactory. In all analyses given below the wet method as described was used. In the ashing of tissue a preliminary digestion with fuming nitric acid was carried out. Usually 50 cc. of acid was added and allowed to stand some hours. Subsequently digestion over a water bath gives complete solution. The solution while warm is transferred to 800 cc. pyrex Kjeldahl flasks, care being taken to secure a complete rinsing of the beaker. Sulphuric acid (20 cc.) is added and the flasks heated to drive off the water and nitric acid. Perchloric acid (8 to 10 cc. of 60 per cent solution) is added and heat applied until the solution is clear. This final solution while still hot is transferred quantitatively to a 200 cc. volumetric flask. After cooling to room temperature the flask is made up to the mark with distilled water. This solution is stored in tightly stoppered flasks and represents the original sample of tissue dissolved in 200 cc. of fluid.

25 to 50 cc. portions of these solutions are now pipetted into 100 cc. pyrex Florence flasks and heated over the open flame until the dense white fumes of sulphuric acid are evolved. This procedure insures the removal of all traces of nitric and perchloric acid or any other oxydizing agent which prevent accurate titration. The solution is now diluted with 20 per cent sulphuric acid which has previously been boiled 2 hours, cooled in presence of carbon dioxide and stored in a flask protected from the air by a blanket of pure carbon dioxide. These specimens are now ready to be titrated electrometrically with dilute titanous sulphate solution protected from the air by means of carbon dioxide gas. The

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apparatus and method as described by King and Washburne (2) was used. We did not use the mercury seals at the top of the burette and on the flask containing the titanous sulphate solution as described by these authors; tight fitting rubber stoppers were satisfactory for our purposes. In place of the ferrous ammonium sulphate standard, as used by the authors, a solution of iron wire in hydrochloric acid was prepared. One-tenth of a gram of iron wire c.p. is accurately weighed and dissolved in dilute hydrochloric acid. A small amount of 30 per cent hydrogen peroxide is added to insure complete oxidation. The solution is now diluted to 2000 cc. Twenty cc. portions are now pipetted into 100 cc. beakers and allowed to concentrate slowly on the steam bath. When the volume approaches 5 cc. the samples are transferred to 100 cc. pyrex Florence flasks with the specially prepared sulphuric acid and titrated with titanous sulphate using the apparatus referred to above. One troublesome source of error, resulting in failure to get checks on different iron standard specimens, often occurs if the standard is allowed to concentrate below a volume of about 5 cc., due to the loss of iron by volatilization. Five cc. of concentrated sulphuric acid may be added and the standard heated down over the open flame in the same manner as the tissue specimens are heated. Two determinations were made upon each specimen and in some cases two separate digestions of the same organ were checked. The method although cumbersome proved to be quite accurate and dependable even for very small amounts of iron.

EXPERIMENTAL OBSERVATIONS

We believe that in our hands the method employed in these dogs prepares tissues and viscera which are practically free from blood— let us say 1 per cent of normal or less. The spleen and bone marrow are exceptions but even here the tissues contain relatively few red cells and those cells are largely in the intersinusoidal stroma. With anemia we note that the spleen and bone marrow show very low iron figures as compared with normal controls (Table 52) and this gives support to our belief that even in these organs the method of vivi-perfusion gives a pretty complete washing out of mature red cells.

The iron content of colon, jejunum, stomach and urinary bladder was determined on seventeen of the dogs and is not included in the tables. The fresh specimens include the mucosa. The content of the *stomach* varied from 0.9 mg. to 2.1 mg. per 100 gm. of fresh tissue averaging about 1.5 mg. The content of the *jejunum* varied from 0.9 mg. to 3.3 mg. with an average content of 1.7 mg. The lowest value for the colon was 0.8 mg. per 100 gm. of fresh tissue in Dog 30-174 which was fed on a diet of anemia bread and milk for

31 days (Table 51). The highest values for the colon were 4.5 mg. in Dog 29-288 which presented an unexplained hemoglobinuria (Table 52) and 3.7 mg. in Dog 30-125 which received 500 mg. of iron daily as iron citrate added to the kennel diet. The average for the *colon* was 2.2 mg. per 100 gm. of fresh tissue. The iron content of the urinary bladder averaged 2.2 mg. per 100 gm. of fresh tissue. These figures indicate that the *gastrointestinal tract* although concerned with the absorption and excretion of iron in its varied combinations, is not concerned with storage of any iron compound. These low values also give an admirable control of the viviperfusion method as it is not possible to free the intestinal villi of red blood cells by any type of gravity perfusion. These analyses serve also as controls for the method of iron analysis.

When the experiments in Table 51 were planned we did not realize that our stock dogs with high blood hemoglobin (140 to 150 per cent) presented a very considerable iron storage as compared with anemic dogs (Table 52). In the face of this conspicuous iron storage in the dogs taken out of stock (Dogs X-2806 and X-2884, Table 51) we hesitate to stress the findings observed in other widely different diets. For a comparison with the iron staining pigment refer to Table 43, Paper IV. It is apparent that the ideal diet experiment would be preceded by a 2 to 3 months' anemia period due to bleeding, at a hemoglobin level of 1/3 normal (40 to 50 per cent hemoglobin).

The first two experiments in Table 51 (Dogs 30-174 and 30-250) were continued on a diet of anemia bread and milk for 31 and 21 days respectively. The make up of this bread has been described elsewhere (3). This diet permits of a minimal regeneration of new hemoglobin in anemia due to bleeding. The iron analysis of various organs is close to the average values.

The third and fourth experiments in Table 51 (Dogs 30-125 and 30-103) were continued 31 and 30 days on a liver diet and diet rich in iron citrate. Both these diets are very favorable to rapid regeneration of new hemoglobin in anemia due to bleeding. The liver diet dog shows average values for all tissues except the liver and spleen. The spleen is above the average if we exclude from the average the dog fed iron citrate. The liver iron analysis is only slightly above the average. We do not attach any significance to these figures.

The dog fed large amounts of iron citrate (Dog 30-125) does show an unusually high iron content for the spleen but the liver content is below normal. The lung also shows a high iron analysis. It is possible that the iron feeding did effect this iron storage.

V. IRON CONTENT OF TISSUES AND VISCERA

The dog fed carrot and mush (30-270, Table 51) for 25 days received a diet unfavorable for new hemoglobin regeneration in anemia due to bleeding but rich in carotin. This healthy airedale shows such high iron values for his liver, spleen and bone marrow that we suspect some unknown condition leading to blood destruction in the weeks or months preceding this experiment. This experiment is entirely out of accord with the other diet dogs and we do not feel that these changes are in any way related to the diet.

TABLE 51
Diet Factors and Iron Content of Perfused Tissues

Dog No.	Type of diet	Duration	Iron in mg. per 100 gm. of fresh tissue								
			Kidneys	Liver	Spleen	Mes. lymph glands	Ribs	Heart	Psoas muscle	Lung	Pancreas
		days									
30-174	Anemia Bread and milk	31	4.7	20.4	38.0	—	22.8	6.5	6.3	10.2	2.4
30-250	Anemia Bread and milk	21	6.9	22.7	40.1	8.3	20.5	3.7	2.8	6.7	2.5
30-125	Fe (500 mg.) + kennel	31	4.0	17.1	86.8	9.7	17.5	3.3	4.3	13.4	1.6
30-103	Liver (300–450 gm.) + meat + bread	30	5.4	30.3	46.1	5.5	14.4	4.0	2.4	6.1	2.0
30-270	Carrot + mush	25	5.0	42.2	53.6	—	14.9	—	3.9	5.5	3.2
X-2806	Kennel	30+	2.2	13.9	23.3	4.8	7.9	3.0	3.4	3.7	1.6
X-2884	Kennel	30+	4.0	25.7	37.8	3.7	7.4	2.9	3.1	2.7	1.3
Average............................			4.6	24.6	46.5	6.4	15.0	3.9	3.7	6.9	2.1

The last two dogs (X-2806 and X-2884, Table 51) are healthy stock dogs which had been on the kennel diet of mixed hospital food scraps for more than 1 month. This diet is moderately favorable for new hemoglobin regeneration in anemia due to hemorrhage. The iron analyses for the tissues are slightly below the general average for Table 51.

Table 52 shows that in anemia the iron content of all tissues and viscera is reduced to minimal levels. The low values for iron are conspicuous in the pancreas and this again serves as an admirable check on the methods of perfusion and iron analysis. For the study of the iron staining pigment in these dogs (Table 52) refer to Table 44, Paper IV. It is of interest that the muscle tissue of the heart

and psoas show no significant change from the control samples.   It is probable that much of this iron is a part of the muscle hemoglobin.

The first two experiments (Table 52) are examples of a pretty severe anemia due to bleeding continued for 1 and 1.5 months respectively.   Even this short anemia period removes practically all the iron reserve storage in spleen, liver and bone marrow.   The kidney iron level is about 1/2 normal for the controls. The liver is reduced to 5 and 4 mg. iron level which is about 1/5 normal for the controls.   The spleen and bone marrow are reduced to about 1/4 and 1/3 normal iron content but part of this iron is surely contained in red blood cells not removed by the viviperfusion.

The third experiment in Table 52 deals with an unusual case of spontaneous hematuria in a dog otherwise normal and healthy.   This dog was under observa-

TABLE 52

Hemorrhage, Anemia and Iron Content of Perfused Tissues

Dog No.	Anemia		Iron in mg. per 100 gm. of fresh tissue								
	Hb. level	Dura-tion	Kid-neys	Liver	Spleen	Mes. lymph glands	Ribs	Heart	Psoas muscle	Lung	Pan-creas
	per cent	*mos.*									
30-271	50–60	1.0	1.4	5.0	17.5		4.1	2.9	4.0	3.3	0.7
30-257	50–60	1.5	2.9	4.0	6.6		3.5	2.5	3.3	2.1	1.1
29-288	95–100	*3.0±	3.0	13.8	15.3	9.4	6.4	2.8	7.8	4.1	1.0
29-8	95–100	**1.0±	3.9	11.4	52.0	8.3		2.9	4.3	5.2	1.3
Average............			2.8	8.6	22.8	8.8	4.7	2.8	4.8	3.7	1.0

* Spontaneous hematuria.
** Closed sterile bile fistula for 9 months.

tion for over 3 months and his blood hemoglobin level was about 2/3 normal (95 to 100 per cent hemoglobin).   Autopsy did not reveal the bleeding spot and all tissues were normal.   Under these conditions the iron reserve storage is not reduced to minimal levels but is distinctly subnormal.

The last experiment in Table 52 concerns an animal with a closed sterile bile fistula which during the last month of life developed an obstruction in the tube and a mild grade of secondary anemia.   The high values for iron in the spleen are probably related to icterus and blood destruction.

Table 53 shows the result of hemoglobin injections of various sorts and in varying amounts both superthreshold and subthreshold, with and without hemoglobinuria.   The organs involved in the reaction

The dog fed carrot and mush (30-270, Table 51) for 25 days received a diet unfavorable for new hemoglobin regeneration in anemia due to bleeding but rich in carotin. This healthy airedale shows such high iron values for his liver, spleen and bone marrow that we suspect some unknown condition leading to blood destruction in the weeks or months preceding this experiment. This experiment is entirely out of accord with the other diet dogs and we do not feel that these changes are in any way related to the diet.

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	per cent	*mos.*									
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29-288	95–100	*3.0±	3.0	13.8	15.3	9.4	6.4	2.8	7.8	4.1	1.0
29-8	95–100	**1.0±	3.9	11.4	52.0	8.3		2.9	4.3	5.2	1.3
Average			2.8	8.6	22.8	8.8	4.7	2.8	4.8	3.7	1.0

* Spontaneous hematuria.
** Closed sterile bile fistula for 9 months.

tion for over 3 months and his blood hemoglobin level was about 2/3 normal (95 to 100 per cent hemoglobin). Autopsy did not reveal the bleeding spot and all tissues were normal. Under these conditions the iron reserve storage is not reduced to minimal levels but is distinctly subnormal.

The last experiment in Table 52 concerns an animal with a closed sterile bile fistula which during the last month of life developed an obstruction in the tube and a mild grade of secondary anemia. The high values for iron in the spleen are probably related to icterus and blood destruction.

Table 53 shows the result of hemoglobin injections of various sorts and in varying amounts both superthreshold and subthreshold, with and without hemoglobinuria. The organs involved in the reaction

to hemoglobin injections with consequent iron storage are the kidney, spleen, liver and bone marrow.

For the clinical histories, data related to the hemoglobin injections and pigment study in these same dogs, refer to Table 41, Paper IV, and accompanying short histories.

The first two dogs in Table 53 (29-252 and 30-135) were given superthreshold hemoglobin doses of dog hemoglobin. The first dog received a few very large

TABLE 53

Hemoglobin Injections and Iron Analyses of Perfused Body Tissues

| Dog No. | Type | Hemoglobin injected | | | Iron in mg. per 100 gm. of fresh tissue | | | | | | | | |
		Mg. per kilo daily	Total	Days inj.	Kidneys	Liver	Spleen	Mes. lymph glands	Ribs	Heart	Psoas muscle	Lung	Pancreas
			gm.										
29-252	Dog	625	31	7	44.8	37.5	177.1	13.8	24.7	3.9	2.5	4.7	3.2
30-135	Dog	125	56	25	17.4	36.8	122.7	22.6	11.9	3.4	4.0	8.4	1.4
29-213	Dog	73	32	29	11.0	27.2	40.2	15.4	11.0	5.1	4.9	3.6	2.3
30-84	Dog	35	48	85	6.2	45.3	101.9	—	41.5	3.7	4.5	7.0	3.1
29-219	Sheep	147	32	15	32.7	30.7	144.6	30.6	64.4	3.5	3.8	5.5	6.3
29-228	Sheep	85	42	17	25.7	34.3	58.7	—	35.6	4.3	3.6	5.6	2.5
30-108	Goose	124	10	15	35.2	20.9	111.8	—	28.6	4.1	2.2	4.5	1.6
30-231	Goose	80	15	15	17.4	21.1	43.8	10.0	—	4.3	2.5	—	1.6
30-149	Muscle	17	3.6	15	14.1	31.4	107.6	—	29.5	4.2	5.9	10.1	3.5
30-228	Muscle	12	2.5	16	9.1	26.6	143.5	—	25.5	4.1	4.8	4.9	1.9
Average....................................					21.4	31.2	105.2	18.5	30.3	4.1	3.9	6.0	2.7

doses with conspicuous hemoglobinuria. The second dog received only a few superthreshold doses at the end of the series and this accounts for the great difference in the renal iron content. The values for spleen and liver are maximal.

The third experiment (Dog 29-213, Table 53) shows a long series of hemoglobin injections very close to the glomerular hemoglobin threshold. There was no iron staining pigment in the renal tubules but the iron analysis is above the control kidney figures so that it is probable that a little hemoglobin seeped through occasionally. The iron figures for the spleen and liver are somewhat below the average. Considering the large amount of hemoglobin injected and the low spleen values one suspects the possibility that the iron reserve storage had been depleted because of unknown factors before this period of observation.

The fourth experiment (Dog 30-84, Table 53) is of especial interest because

this dog received many hemoglobin injections during 3 months to a considerable total but each injection was well below the minimal renal or glomerular threshold for hemoglobin. The kidneys show a control level for iron content while the spleen, liver and bone marrow show the expected large reserve store of iron.

The fifth and sixth experiments (Dogs 29-219 and 29-228, Table 53) show the effect of injections of sheep hemoglobin in considerable total amounts. Both dogs were given superthreshold doses with consequent hemoglobinuria. There is abundant reserve storage of iron in the kidney, liver, spleen and bone marrow. The unusually high figures for the spleen and bone marrow in Dog 29-219 might suggest a less satisfactory viviperfusion than the average.

The seventh and eighth experiments (Dogs 30-108 and 30-231, Table 53) show the reaction to injections of goose hemoglobin, one a superthreshold and the other very close to the minimal renal threshold. The latter (Dog 30-231) shows a low iron figure for the kidney yet above the control average which may indicate an occasional seepage of hemoglobin into the tubules.

The last two experiments in Table 53 deal with muscle hemoglobin injections which caused definite hemoglobinuria. As has been pointed out in Paper IV, muscle hemoglobin is different in several respects from blood hemoglobin. The preparation of muscle hemoglobin has been described elsewhere (4). The final product obtained by passage through a medium Berkefeld filter is crystal clear, having the usual hemoglobin color, and quite sterile. It may be preserved a few days in the ice box but the sooner it is used the better. As muscle hemo-globin ages it does not change in its appearance but does change in some of its physical aspects. When used fresh this solution of muscle hemoglobin is non-toxic to most dogs but with aging in the ice box it develops toxicity. Because the preparation of muscle hemoglobin is quite time consuming there is always a tendency to use the solution for several days rather than prepare new solutions. Sometimes fatal shock is produced. When non-lethal shock is produced one may suspect that hemolysis or injury of red cells may follow. We are inclined to explain the high figures for iron analysis in the spleen on this basis as it is obvious that the iron contained in the few grams of muscle hemoglobin injected cannot account for the large surplus iron storage in the spleen.

DISCUSSION

The importance of the *kidney* in relation to iron conservation comes out clearly in the experiments tabulated above. Under normal con-ditions in a healthy dog the blood free kidney contains about 4.6 mg. iron per 100 gm. fresh tissue. This normal level is reduced to 2.8 or less by short anemia periods. When there is escape of hemoglobin in the blood stream the kidney plays its most important rôle. The kidney glomeruli establish the minimal renal threshold for hemo-globin and prevent its escape so that various phagocytic cells may

completely conserve and remove it from the circulation. Above this minimal threshold the tubular renal epithelium picks up this hemoglobin from the tubular lumen and saves it for future use. When the hemoglobin escape is very large or the renal epithelium is stuffed to repletion, we observe the escape of hemoglobin in the urine. This conservation of iron following superthreshold doses of hemoglobin may raise the iron content of the kidney from normal or 4.6 mg. to 35 to 45 mg. per 100 gm. fresh tissue.

The *liver* is without doubt the most important organ in the conservation and utilization of iron in the body. One may look upon the liver as a warehouse in which the turn over is active and the surplus storage considerable but held within fairly narrow limits. Because of its size the liver contains the largest amount of stored iron although the spleen contains more per 100 gm. fresh tissue. The blood free liver normally contains about 25 mg. iron per 100 gm. fresh tissue with maximal figures of 42 and minimal of 14. Short periods of anemia will reduce this surplus store to 4 or 5 mg. iron per 100 gm. fresh tissue. Long periods of hemoglobin injection do not increase the liver storage of iron to any remarkable extent and we observe average values of but 31 mg. iron per 100 gm. fresh tissue. This is in striking contrast to the spleen (Table 53).

The *spleen* in contrast to the liver we look upon as a favored storehouse for iron but the turn over here is probably much slower than in the liver. Much of the iron in the spleen is in the form of iron staining pigment (Paper IV). The iron storage in the spleen can be notably depleted by anemia and in certain cases (Table 52) may even approximate the liver values. In fact during periods of continued anemia in the dog all the stores of iron are drawn upon and conspicuous depletion is obvious in spleen, liver and bone marrow.

The *red marrow* of the ribs is of considerable interest when compared with the spleen and runs a remarkably close parallel to it. Ribs in the adult dog consist of bone and red marrow, the latter making up 1/3 to 1/2 the total volume. If we figure the marrow as 1/3 by weight and compare rib marrow and spleen, the parallelism is remarkably close. In three anemia dogs, excluding the bile fistula (Table 52) the iron reads 4.7 mg. for marrow and 13 mg. for spleen. In the normal group (Table 51) the iron analyses read 15 mg. for

the marrow and 39.8 mg. for the spleen, excluding the high spleen value in the iron feeding experiment (Dog 30–125). In the group injected with hemoglobin (Table 53) the red marrow reads 30.3 mg. and the spleen 105 mg. iron per 100 gm. fresh tissue.

In histological study of the spleen and marrow (Paper IV) it was found that the iron staining pigment appeared to be identical in general distribution in both tissues so that the spleen tabulation was considered representative of both spleen and marrow. The mesenteric and retroperitoneal lymph glands show relatively little of interest. The histological study (Paper IV) shows the presence of iron staining pigment when there is evidence that pigment was being deposited in related viscera whose lymph drainage is carried through the lymph glands studied. During anemia periods this iron store is drawn upon but probably more slowly even than the spleen. The small amount of tissue makes analysis relatively inaccurate. In normal dogs this gland tissue assays about 7 mg. iron per 100 gm. fresh tissue as compared with 18 mg. iron in the hemoglobin injected dogs. The amount of iron concerned in lymph gland tissue therefore is trivial.

Heart muscle and *skeletal muscle* (psoas) show great uniformity of iron content. The figures for iron in heart or skeletal muscle run close to 4 mg. per 100 gm. fresh tissue whether the dog is normal or has received a series of hemoglobin injections. In anemia the heart muscle may show a slight decrease. It is probable that a large part of this iron is within the muscle hemoglobin molecule which is an essential part of these muscle fibres.

The *lungs* show a low iron content which fluctuates somewhat from a minimum of 2.7 to a maximum of 10 mg. per 100 gm. fresh tissue with average values of 6 to 7 mg. in dogs which are normal whether they have been injected with hemoglobin or not. During anemia the iron content falls somewhat to an average of 3.7 mg.

The *pancreas, stomach, jejunum, colon* and *urinary bladder* all present minimal values for iron. The low normal values for the pancreas (2.1 mg. iron) are increased very slightly by hemoglobin injections (2.7 mg. iron) and depleted by anemia to minimal tissue levels (1.0 mg. iron). The stomach shows average values of 1.5 mg. iron, the jejunum 1.7 mg. iron and the colon 2.2 mg. iron. The iron content of the urinary bladder is 2.2 mg. iron per 100 gm. fresh tissue.

completely conserve and remove it from the circulation. Above this minimal threshold the tubular renal epithelium picks up this hemoglobin from the tubular lumen and saves it for future use. When the hemoglobin escape is very large or the renal epithelium is stuffed to repletion, we observe the escape of hemoglobin in the urine. This conservation of iron following superthreshold doses of hemoglobin may raise the iron content of the kidney from normal or 4.6 mg. to 35 to 45 mg. per 100 gm. fresh tissue.

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These last figures indicate that *smooth muscle* contains very little iron. The same statement applies to the *mucosa* of the stomach, jejunum and colon which was a part of the tissue used for analysis.

SUMMARY

When hemoglobin is set free in the circulation the *kidney* plays an important part in the *conservation of iron*. When the renal threshold is not exceeded by the hemoglobin in the blood there is little or no excess iron deposited in the kidney but when superthreshold doses of blood hemoglobin are given the epithelium of the convoluted tubules accumulates much iron and the iron analyses may show 5 times normal values.

The normal dog (140 to 150 per cent hemoglobin) has a large reserve store of iron in the liver, spleen and marrow. Diets may modify this storage of iron in these tissues. To bring conclusive proof relating to the individual diet factors, the reserve store of iron should be depleted by an anemia period of 2 to 3 months.

Complete removal of red cells from tissue capillaries is essential for accurate iron assays of fresh tissue. The method described accomplishes this without causing gross tissue edema.

The lowest iron content is observed in the pancreas, stomach, jejunum, colon and urinary bladder. These figures average from 1 to 2 mg. iron per 100 gm. fresh tissue. This shows that smooth muscle and mucous membranes contain little iron.

Striated muscle (heart, psoas) shows a relatively low iron content but uniform values close to 4 mg. per 100 gm. tissue.

Lungs show a considerable fluctuation with low iron values in anemia (3.7 mg.) and higher values in health (6 to 7 mg.).

The *spleen* shows maximal fluctuations and the highest reserve storage of iron per 100 gm. fresh tissue. The spleen iron analyses show low values in anemia (7 to 15 mg.) and wide differences in controls (25 to 50 mg.). With hemoglobin injections the iron storage is conspicuous and iron analyses may run as high as 150 to 175 mg. iron per 100 gm. fresh tissue.

Bone marrow of the rib runs in parallel with the spleen as regards iron storage following hemoglobin injections and depletion following anemia periods.

· The *liver* because of its weight always contains the main bulk of the iron stored in the blood free tissues of the body. Its store is depleted by anemia even to levels of 4 to 5 mg. iron per 100 gm. fresh tissue. In the normal dog the iron store in the liver averages 25 mg. per 100 gm. tissue. Frequent hemoglobin injections may increase this level to 31 mg. iron per 100 gm. The liver is considered the most active clearing house for iron storage and utilization.

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STUDIES IN THE SEROLOGY OF SYPHILIS

VI. The Induction of Antibodies to Tissue Lipoids (A Positive Wassermann Reaction) in Normal Rabbits

By HARRY EAGLE, M.D.[*]

(From the Syphilis Division of the Medical Clinic of The Johns Hopkins University, Baltimore)

Contrary to the traditional view that proteins are the only type of substance with antigenic properties, it has been recently found that under certain conditions specific antibodies may be produced in the experimental animal to both lipoids and carbohydrates. Thus, Forssman (1911) found that guinea pig kidneys, injected into rabbits, induce the formation of antibodies to a lipoid present in the kidneys. As Landsteiner and Simms showed (1921, 1923), this lipoid is non-antigenic when injected as such, but when mixed with a foreign protein (*e.g.* pig serum) before injection, it induces the formation of the characteristic antibody. To this type of "incomplete" antigen, which can combine with a specific antibody, but which cannot stimulate its formation without the aid of a foreign protein, Landsteiner gave the name "haptene."

Sachs, Klopstock, and Weil (1925) have obtained similar results with the tissue lipoid used as "antigen" in the Wassermann reaction. Although rabbit lipoid alone is non-antigenic for rabbits, they found that when it is injected together with a foreign protein (pig serum), antibodies are formed against the lipoid; *i.e.*, the rabbit develops a positive Wassermann reaction. Because of this observation, they have suggested that the Wassermann reaction in syphilis is an antibody response to autogenous lipoids liberated at foci of infection and tissue destruction. True, human lipoids as such are non-antigenic; but they draw an analogy to the animal experiment and suggest that

positive frequently give a positive precipitation test with tissue lipoid antigens; more significant, upon the removal of this normal antibody to lipoid by absorption of the serum with a beef heart antigen, the Wassermann and precipitation tests both become completely negative (Protocol 2).

Protocol 2.—To 2 cc. of Wassermann positive normal rabbit serum was added 0.4 cc. of beef heart flocculation-antigen suspension.[1] After 4 hours at 37°C., 3.6 cc. of NaCl N/7 were added, the mixture (*a*) centrifuged, (*b*) passed through a Berkefeld filter, and the clear supernatant fluid (1:3 serum) tested for its reagin content as outlined in the following tabulation, using the technique described in Protocol 1.

Serum, *cc*	0.2	0.1	0.05	0.025	0.0125	0.0062
(*a*) Complement fixation by supernatant fluid obtained after centrifugation	+	±	0	0	0	0 (anticomplementary in first two tubes)
(*b*) Complement fixation by Berkefeld filtrate of absorbed serum	±	0	0	0	0	0
Complement fixation by untreated serum, 1:3	+	+	+	±	0	0
Precipitation test, absorbed serum	0	0	0	0	0	0
Precipitation test, untreated serum, 1:3	+	+	±	0	0	0

As already stated, we believe the discordant findings in the literature can be reconciled on the basis of the differences in the Wassermann techniques employed. The reagin of normal rabbit serum, present in one-half to two-thirds of 100 animals tested by the technique of Protocol 1 can be masked in whole or in part (1) by using fresh serum, (2) by using a non-cholesterolized antigen, (3) by incubating for only $\frac{1}{2}$ hour at 37°C., or (4) by using an excess of serum containing native amboceptor.

[1] The method of preparation of this antigen is to be described in a forthcoming paper. The same result is obtained with the suspension formed in diluting a cholesterolized (0.6 per cent) beef heart antigen with an equal volume of NaCl N/7.

To be sure, this Wassermann reaction of normal rabbits differs materially from that observed in human syphilis. The reactions are not as clear-cut, there being frequently incomplete hemolysis and fixation; the aggregates in the precipitation test are difficult to see; the two types of test do not parallel each other as consistently as they do in human syphilis; finally, rabbit sera give this same type of weak complement fixation with many lipoid suspensions which do not possess any reactivity with syphilitic serum. Nevertheless, in using the rabbit as an experimental animal for the study of the Wassermann reaction, this pseudo-fixation given by normal rabbit sera, however unlike the fixation as given by syphilitic sera, must be constantly kept in mind as a potential source of error. In testing the effect of any particular procedure upon the Wassermann reaction of rabbits, it is essential to titrate the sera quantitatively, and to include some normal rabbit sera in every Wassermann series.

Lipoids and Lipoid-Protein Compounds as Antigens

A. The Effect of Lipoids Alone.—

1. Rabbit, beef, and human hearts were extracted by the method described in Protocol 1, Paragraph 2. The yellow residues obtained by evaporating the alcohol extracts to dryness were resuspended in a minimum volume of NaCl N/7. Amounts corresponding to 12 cc. of extract were injected intravenously into rabbits three times a week for 3 to 4 weeks. Three rabbits were injected with each type of lipoid. Of the nine rabbits, four died in the course of the injections; the remaining five showed no significant changes in their Wassermann titre at any time during or after the course of injections.

2. Nine rabbits were similarly injected with suspensions of dried cholesterolized (0.6 per cent) antigens, again with negative results.

3. Each of the three alcoholic heart extracts were concentrated to one-fifth their original volume, and each extract dropped slowly, with shaking, into four volumes of NaCl N/7. 10 cc. of the milky stable suspensions formed were slowly injected intravenously. Five rabbits were used for each type of antigen, a total of 15 rabbits in all. As before, injections were also carried out with cholesterolized (0.6 per cent) extracts. The results observed in the surviving animals are given in Table I.

The results can be briefly summarized as follows: Rabbit lipoid, no matter how dispersed, does not produce antibodies when injected into rabbits intravenously. Beef or human lipoid is also non-antigenic

TABLE I

Response of Rabbits to Intravenous Injections of Colloidally Dispersed Lipoid.

Source of lipoid	Type of extract	Wassermann titre* before injections	Wassermann titre after 3 wks. injections
Rabbit heart..................	Plain alcoholic extract (ether-insoluble)	32	24
		0	0
		24	8
		24	32
	Cholesterolized alcoholic extract	8	8
		32	32
		32	24
Beef heart..................	Plain alcoholic extract	0	32
		16	16
		6	16
		24	24
		32	96
	Cholesterolized alcoholic extract	64	96
		8	4
Human heart...............	Plain alcoholic extract	24	16
		24	96
		32	16
	Cholesterolized alcoholic extract	16	128
		24	96
		0	48
		32	24

* The method used in estimating the reagin titre is illustrated by the following example:

Serum, cc.	0.2	0.1	0.05	0.025	0.0125	0.0062
Wassermann result.	+	+	+	+	+	±
Dilution of serum.	1:1	1:2	1:4	1:8	1:16	1:32

0.0062 cc., *i.e.* 0.2 cc. of a 1:32 dilution, gave a ± result; 0.2 cc. of a 1:16 dilution gave a + result. The maximum dilution of which 0.2 cc. gave a positive result was therefore approximately 1:24, a reagin titre of 24. The sources of error inherent in the Wassermann reaction do not justify any finer approximation. Any change of < 200 per cent is to discarded as being within the limits of experimental error.

when the dry material is crudely suspended in NaCl N/7; but a colloidally dispersed suspension does seem to have some slight antigenic activity. In the light of the subsequent experiments, it is possible that this antigenicity rests upon the presence in the extracts of traces of beef and human protein. Too much stress should not be laid on the positive results of Table I, as the serum of normal rabbits may contain from 0 to 64 reagin units, while the highest titre induced by colloidally dispersed lipoid was 128 units.

B. Lipoid-Human Serum Mixtures as Antigens.—

The cholesterolized (0.6 per cent) alcoholic extracts of rabbit, beef, and human hearts were evaporated down to one-third their original volume, and dropped slowly with shaking into ten volumes of 1:10 normal Wassermann negative human serum. After standing for 1 hour at 37°C., 15 cc. of each mixture were injected intravenously into each of three rabbits. Injections were repeated three times a week for 3 weeks, the Wassermann reaction being tested each week for 1 month. The results are given in Table II. As rabbits died, they were replaced.

TABLE II

Effect of Lipoid-Human Serum Mixtures upon Reagin Titre of Rabbits

Antigen injected	Animal No.	Original reagin titre	Reagin titre after wk. No.				Maximum reagin titre / Original titre	Average increase
			1	2	3	4		
Cholesterolized beef heart lipoid + normal human serum	1	32	24	48	128	64	128/48	per cent
	2	24	32	24	96	24	96/24	
	3	0	0	0	48	48	48/0	300
Cholesterolized human heart lipoids + normal human serum	1	16	16	16	96	128	128/16	
	2	48	24	32	128	64	128/24	
	3	24	32	48	192	48	192/24	400
Cholesterolized rabbit heart lipoid + normal human serum	1	8	8	8	48	24	48/8	
	2	32	32	24	128	128	128/32	
	3	0	0	0	0	0	0/0	400
Controls with injections of NaCl N/7	1	48	32	48	48	32	48/48	
	2	16	24	12	16	24	24/16	
	3	24	24	16	48	32	32/24	50

C. The Lipoid-Reagin Precipitate as Antigen.—We have thus verified the finding of Sachs, Klopstock, and Weil that a mixture of foreign protein (*i.e.* foreign to the rabbit) and tissue lipoids, injected into rabbits, produces antibodies against the lipoids. If, as we maintain, the precipitate formed by adding these lipoids to syphilitic serum consists of a firm combination of the lipoid and the reagin-globulin, such a precipitate should be antigenic for rabbits. That such is the case is shown in the following experiment.

The cholesterolized heart extracts (beef, rabbit, and human),[2] prepared as described in Protocol 1, were diluted with an equal volume of NaCl N/7. The milky suspension thus formed was added to ten volumes of strongly Wassermann positive syphilitic serum, previously inactivated at 56°C. for ½ hour. After 24 hours at room temperature, the mixture was diluted with two-third volumes of NaCl N/7, and centrifuged at high speed. The white precipitate was washed repeatedly in NaCl N/7 until the supernatant fluid was protein-free, and finally resuspended in NaCl N/7 to form a heavy milky suspension of approximately half the volume of the antigen used in its preparation. The average solid content of this suspension was 8 to 12 mg. per cc.

2 to 4 cc. of the three suspensions were injected intravenously three times a week for 3 weeks into a total of twenty-five rabbits, quantitative Wassermann and precipitation tests being performed on each rabbit at intervals in the course of 4 weeks after the beginning of injections.

The results are summarized in Table III.

The results were clear-cut; not only did every rabbit develop antibodies to lipoids, but the titres obtained were several times higher than the strongest Wassermann reaction we have ever observed in syphilitic serum, whether of human beings or of rabbits, and many times higher than the titres induced by the injection of lipoid-serum mixtures.

Exception might be taken to the validity of our interpretation of this experiment, on the ground that the lipoid-reagin compound may conceivably dissociate into its component parts when injected into the rabbit. The rabbit's blood might in such a case contain circulating human syphilitic reagin, and not an antibody produced as a response

[2] In keeping with the suggestions embodied in the 1928 report of the League of Nations Conference on the Serodiagnosis of Syphilis, throughout this paper completely positive results, whether fixation or precipitation, are reported +, completely negative results are reported 0, and doubtful or incomplete results are reported ±.

to the antigenic stimulus of the lipoid-reagin mixture. True, in our experience such dissociation cannot be accomplished *in vitro*; furthermore, there is no evidence that it takes place *in vivo*. Nevertheless,

TABLE III

Effect of the Lipoid-Reagin Precipitate When Injected Intravenously into Rabbits

Antigen injected	Animal No.	Original Wassermann titre*	Wassermann titre after wk. No.				Maximum titre* / Original titre	Increase
			1	2	3	4		*per cent*
Beef heart lipoid-human reagin precipitate	1	30	—	—	480	320	480/30	1,600
	2	20	20	—	320	—	320/20	1,600
	3	10	20	40	80	160	160/10	1,600
	4	30	16	48	—	320	320/30	1,000
	5	60	32	48	1,024	158	1,024/60	1,500
	6	0	0	—	512	512	512/0	—
	7	8	—	—	256	1,024	1,024/8	12,000
	8	16	—	—	512	128	512/16	3,000
	9	10	—	—	1,600	400	1,600/10	16,000
	10	20	20	40	1,600	1,200	1,600/20	8,000
	11	64	32	96	1,024	1,024	1,024/64	1,500
	12	32	16	16	128	64	128/32	400
Rabbit heart lipoid-human reagin precipitate	1	10	10	40	320	160	320/10	3,200
	2	0	0	0	320	320	320/0	—
	3	0	0	16	512	256	512/0	—
	4	8	16	8	256	128	256/8	3,000
	5	16	8	64	512	512	512/16	3,000
	6	10	64	80	1,600	640	1,600/10	16,000
	7	24	80	16	1,600	356	1,600/24	7,000
Human heart lipoid-human reagin precipitate	1	16	0	32	1,024	256	1,024/16	6,000
	2	0	32	64	512	128	512/0	—
	3	32	0	32	128	480	480/32	8,000
	4	0	0	480	320	160	320/0	—
	5	0	32	320	1,600	128	1,600/0	1,600
	6	0	0	32	1,600	100	1,600/0	—

* With beef heart lipoid as antigen. Approximately the same results are obtained if either human or rabbit heart lipoid is used in the test.

even granting the possibility that it may take place, the experiment itself affords data which indicate that the antibody demonstrated in the rabbits' sera is not human reagin liberated from its combination

with lipoid, but is an antibody formed *de novo* in response to an anti-
genic stimulus.[3]   These data are as follows:

1. The chronological curve of reagin titre during a series of injec-
tions shows the latent period characteristic of antibody production.
For 1 to 2 weeks there is no significant change in titre, and then,
almost explosively, it multiplies 10-, 20-, and even 50-fold within a
few days.   If this were merely reagin liberated from the injected
precipitate, the curve of its appearance should be roughly linear.

2. The amount of reagin present in the injected rabbits is many
times the total amount of reagin potentially present in the lipoid-
reagin precipitate.   The 18 to 36 cc. of precipitate suspension in-
jected into each rabbit, correspond to a maximum of 1,600 cc. of
syphilitic serum, with an average titre of 25 to 50 reagin units per cc.
Assuming that this is recovered in its entirety by dissociation of the
precipitate *in vivo*, a total of 40,000 to 80,000 reagin units have been
injected.   The maximum titre we have observed after nine injections
is approximately 1,600 units.   Assuming that blood constitutes 7
per cent of the body weight, this rabbit (weighing 3.0 kilos) may be
said to have 210 cc. blood; *i.e.*, about 120 cc. of serum.   The total
reagin content of this blood was therefore approximately 200,000 units.
Remembering that the tissue juices contain antibody and reagin in high
concentration, it is clear that even a 100 per cent dissociation of reagin
from the precipitate injected and a 100 per cent retention in the animal
body would account for only a small fraction of that present in this
rabbit.

3. If we assume that the dissociation of reagin takes place with a
precipitate derived from a foreign serum, it should occur as readily
with antibody derived from homologous serum.   Actually, however,
90 cc. of a lipoid-reagin precipitate suspension, obtained from a rabbit
antiserum and representing approximately 200,000 reagin units, failed
to cause any significant increase in the Wassermann titre of another
rabbit when injected intravenously over a period of 3 weeks (see
Protocol 4).

[3] It should be noted that no matter what type of lipoid was used in the prepara-
tion of the precipitate, whether human, rabbit, or beef, the antibody formed
reacts equally well with all three.   There is no evidence of species specificity.
In this respect the antibody is like the reagin of syphilitic serum.

4. Finally, the antibody which appears as the result of the injecti differs materially from the reagin of human syphilitic serum. though the latter reacts equally well with a human, beef, or rab lipoid, it fails to react with alcoholic extracts of milk, of human sheep red cells, or with solutions of lecithin; the antibody induced rabbits, however, is characterized by a surprising lack of specificity It gives complement fixation with every one of the above antigens as well as with finely divided suspensions of cholesterol and sitosterol (Protocol 3, Table IV).

Protocol 3. The Antibody Response to Tissue Lipoids Is Not Specific.—(1) Dried sheep cells, (2) human cells, and (3) skimmed milk powder were extracted three times with ether (4 cc. per gm.), and the dried residue extracted for 2 weeks with 95 per cent alcohol. The alcoholic extract was concentrated to one-third its original volume, and fortified with 0.6 per cent cholesterol. (4) Lecithin (Merck) was dissolved in 95 per cent alcohol to form a 2 per cent solution, which was then fortified with 0.6 per cent cholesterol. (5) Cholesterol and (6) sitosterol were dissolved in 95 per cent alcohol to form a 1 per cent solution.

The sera of (1) normal rabbits, of (2) rabbits injected with the lipoid-reagin precipitate, and of (3) syphilitic human beings were tested for complement fixation with each of these antigens, care being taken to use the antigen in a dilution sufficiently removed (< 1/8) from the anticomplementary concentration to ensure against technical false positive reactions. The technique was otherwise the same as that described in Protocol 1. Only one experiment is here given in detail. Qualitatively similar results were obtained in eight similar comparative tests.

As is seen in Table IV the injections cause an increase in the complement-fixing titre of the rabbit serum against all the lipoids listed; the antibody formation could therefore not be due to the liberation *in vivo* of human reagin which reacts only with the beef heart lipoid.

It follows from these lines of evidence that the large quantities of antibody to tissue lipoids present in the rabbit serum after the injection of a (beef, rabbit, human) lipoid-reagin precipitate are formed *de novo* in the rabbit, and are not due to dissociation in the animal of the human reagin present in the precipitate. This result furnishes an additional verification of our original thesis that the precipitation of lipoids by syphilitic human serum is due to the deposition of a sensitizing globulin film around the individual lipoid particles. As in the experiments of Sachs and of Landsteiner, this foreign (human) protein confers antigenicity upon the lipoid; but the reagin-globulin film,

TABLE IV

The Antibody Response in Rabbits to Tissue Lipoids Differs from the Reagin of Syphilitic Human Serum

Type of serum	Dilution of serum	Complement fixation with							Anticomplementary control
		Beef heart extract 1:40	Cholesterol 1:100	Sitosterol 1:100	Lecithin 1:8,000	Milk 1:200	Sheep cell 1:800	Human cell extract 1:100	
Normal rabbit serum	1	+	±	+	+	+	+	+	0
	1:2	+	±	±	+	+	+	+	0
	1:4	+	±	±	+	+	+	+	0
	1:8	+	0	0	±	+	+	+	0
	1:16	0	0	0	0	+	+	0	0
	1:32	0	0	0	0	+	+	0	0
	1:64	0	0	0	0	±	0	0	0
	1:128	0	0	0	0	0	0	0	0
	1:256	0	0	0	0	0	0	0	0
	1:512	0	0	0	0	0	0	0	0
	1:1,024	0	0	0	0	0	0	0	0
Serum titre.......		8	4	4	6	48	32	8	0
Rabbit serum after 9 injections with rabbit heart lipoid-human reagin precipitate	1	+	+	+	+	+	+	+	+
	1:2	+	+	+	+	+	+	+	+
	1:4	+	+	+	+	+	+	+	+
	1:8	+	+	+	+	+	+	+	+
	1:16	+	+	+	+	+	+	+	+
	1:32	+	+	+	+	+	+	+	+
	1:64	+	+	+	+	+	+	+	0
	1:128	+	+	+	+	+	+	+	0
	1:256	+	+	+	+	+	+	+	0
	1:512	+	0	0	+	+	+	0	0
	1:1,024	0	0	0	0	0	±	0	0
Serum titre.......		500	250	250	500	500	750	250	32
Syphilitic human serum	1	+	0	0	· 0	0	+	0	0
	1:2	+	0	0	0	0	+	0	0
	1:4	+	0	0	0	0	±	0	0
	1:8	+	0	0	0	0	0	0	0
	1:16	+	0	0	0	0	0	0	0
	1:32	+	0	0	0	0	0	0	0
	1:64	+	0	0	0	0	0	0	0
	1:128	0	0	0	0	0	0	0	0
	1:256	· 0	0	0	0	0	0	0	0
	1:512	0	0	0	0	0	0	0	0
	1:1,024	0	0	0	0	0	0	0	0
Serum titre.......		64	0	0	0	0	2–4	0	0

firmly anchored to each lipoid particle, is apparently more efficient in this respect than the loosely adsorbed protein of the simple serum-lipoid mixture which they used.

That the foreign protein (human reagin-globulin) plays an essential rôle in conferring antigenicity upon the lipoid with which it has combined is shown by the fact that a similar precipitate obtained from rabbit sera instead of human sera and thus containing rabbit protein instead of human protein is completely non-antigenic for rabbits, even when injected in massive quantities (Protocol 4). Moreover, if the lipoid-human reagin precipitate is heated at 100°C. for 5 minutes, it loses its antigenicity entirely, despite the fact that the lipoid as such is not affected. This is considered to be due to the heat coagulation of the reagin-globulin film.

Protocol 4. A Lipoid-Reagin Precipitate Derived from Rabbit Serum Is Non-Antigenic for Rabbits.—A cholesterolized rabbit heart antigen was diluted with an equal volume of NaCl N/7, and the milky suspension added to five volumes of the rabbit antisera formed as described in Protocol 3. As before, the precipitate was washed and injected in quantities of 1, 2, 5, and 10 cc. three times a week for 3 weeks. No significant changes in Wassermann titre (<50 per cent) were observed in any of the four rabbits.

The same lipoid-reagin precipitate suspension which is so highly antigenic for rabbits is not in the slightest antigenic for guinea pigs; at least, repeated intracardial injections of comparatively enormous quantities (1 to 2 cc. three times a week for 4 weeks) failed to cause any of the four guinea pigs to develop a positive Wassermann reaction. The intravenous route is the only one which is successful even in rabbits; at least, we find that intraperitoneal and subcutaneous injections give uniformly negative results, none of eight rabbits so injected showing any change in Wassermann titre. Although two out of six rabbits injected intramuscularly did develop a slightly increased Wassermann titre, the increase was not sufficiently marked to be considered significant.

It is interesting also to note that rabbits with this high artificially induced Wassermann titre are just as susceptible to intratesticular inoculations with pathogenic *Sp. pallida* as are control animals, judged by the duration of the incubation period and by the size of the lesion produced.

THE MAINTENANCE OF A NORMAL PLASMA PROTEIN CONCENTRATION IN SPITE OF REPEATED PROTEIN LOSS BY BLEEDING

By C. W. BARNETT, M.D., R. B. JONES, M.D., AND R. B. COHN

(*From the Department of Medicine, Stanford University Medical School, San Francisco*)

Two characteristic features of nephritis of the degenerative type are pronounced albuminuria and lowered plasma proteins. The simplest explanation of the plasma protein depletion is, obviously, that regeneration is slow and is unable to keep pace with the albuminuria. The adequacy of this theory could be easily tested if the maximum rate of plasma protein regeneration in normal individuals could be determined. This has not been accomplished in man but has been done in animals.

Kerr, Hurwitz and Whipple (1) studied the regeneration in dogs by noting the time taken for the normal level to be regained after a marked initial depletion. They reduced the protein by the method of plasmapheresis, that is, by the withdrawal of large quantities of blood, followed by the injection of the red cells suspended in Locke's solution after the removal of the plasma. In most of their experiments, over 50 per cent of the total plasma protein was removed and they found that from 7 to 14 days were required for the replacement of the lost protein. Regeneration was only slightly more rapid on a high protein diet than on starvation but was considerably reduced when the liver was damaged. They did not express their results in actual amounts of protein regenerated, but from their protocols, approximate calculations can be made. The highest rate of regeneration in their experiments was about 0.15 gm. per kilo per day. They concluded that the regeneration of plasma protein is a slow process.

Smith, Belt and Whipple (2) repeated this work using a modified bleeding technic which permitted a protein reduction to about one-third of the normal. They studied the curve of regeneration and found an immediate rise within 15 minutes of about 0.5 per cent and a return to normal in from 2 to 7 days. The rates of regeneration were much greater than those observed by Kerr, Hurwitz and Whipple. The average daily regeneration in nine experiments was 0.42 gm. per kilo per day, and the highest, 0.89 gm. per kilo per day.

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to study the rate of regeneration of the various protein fractions, but as they published no protocols, the exact rate of regeneration that they observed cannot be determined.

Leiter (4–6), Barker and Kirk (7) and Shelburne and Egloff (8) used the method of plasmapheresis to lower plasma proteins in the study of edema but did not directly measure the rate of regeneration.

In all the previous work regeneration has been studied when the plasma proteins were reduced to a very low level. We have found no record of any experiments designed to determine the maximum amount of protein that can be removed daily over a considerable period of time without lowering the protein level in the blood. The object of the present experiment was to determine this quantity in dogs.

Experimental Method

A series of normal dogs of approximately the same size were taken and varying quantities of blood were removed daily by aspiration from a jugular vein, the amounts removed being kept as nearly constant as possible in each individual dog. Dry sodium citrate was used as an anticoagulant, the blood was centrifuged, the plasma was drawn off, measured and replaced with Locke's solution, after which the cells were kept in an ice box to be reinjected the following day after the next bleeding. The total plasma proteins were measured gravimetrically after coagulation with heat at a pH of 4.9. The method is similar to that of Starlinger and Hartl (9) except that a half normal acetate buffer is used instead of N/50 acetic acid, and filtration is carried out on asbestos filters instead of filter papers, the rapid absorption of water by a dried filter paper making accurate weighing on an analytical balance impossible.

The procedure used is as follows:

Asbestos filters are prepared using thoroughly washed asbestos on Jena glass filters (1 G 3). These are dried to a constant weight in an incubator at 105°C. Complete drying is usually accomplished in 24 hours. All filters are kept in a desiccator over P_2O_5 when not in the incubator.

Blood is taken using dry sodium citrate as an anticoagulant and the plasma is drawn off after centrifuging. 1 cc. of plasma and 5 cc. of half normal sodium acetate acetic acid buffer with a pH of about 4.9 are placed in a test-tube and immersed in boiling water for 15 minutes. The contents of the tube are then filtered through a weighed asbestos filter with suction, care being taken to transfer all the precipitate to the filter. After the filtrate has been completely drawn through, the filter is washed twice with about 10 cc. of distilled water, twice with 95 per cent alcohol, twice with ether and finally twice with boiling absolute alcohol. The filter is then dried to constant weight at 105°C. This is usually accomplished

in 24 hours, but occasionally 48 hours is required. The number of milligrams of protein on the filter divided by 10 gives the plasma protein in per cent.

The determination of protein gravimetrically seems more satisfactory than by any of the indirect methods of estimation. In the present method there are three principal sources of error. First, there is the loss of protein by incomplete coagulation or faulty filtration, second, the inclusion in the precipitate of salts, and third, the inclusion of non-protein organic substances, principally fat. We have attempted to eliminate these possible sources of error and feel that the method is accurate to within 0.3 gm. per cent or about 5 per cent.

TABLE I

Quantities of Protein Lost in the Filtrate and Wash Water

No.	N in filtrate	Non-protein nitrogen	Protein N in filtrate	Protein in filtrate	Protein in ppt.	Loss
	mg.	mg./cc.	mg.	mg.	mg.	per cent
1	0.21		0.21	1.3	63	2.1
2	0.62	0.35	0.27	1.7	55	3.1
3	0	0.35	0	0	55	0
4	0.39	0.35	0.04	0.25	55	0.5
5	0.50	0.35	0.15	0.9	55	1.6
6	0.52	0.32	0.20	1.2	54	2.2
7	0.52	0.32	0.20	1.2	54	2.2
8	0.25	0.24	0.01	0.06	57	0.1
9	0.26	0.24	0.02	0.12	57	0.2
10	0.32	0.24	0.12	0.8	57	1.4
11	0.23	0.24	0	0	57	0
12	0.60	0.36	0.24	1.5	61	2.5
13	0.55	0.36	0.19	1.2	61	2.0
14	0.45	0.36	0.09	0.6	61	1.0
15	0.35	0.36	0	0	61	0
16	0.42	0.35	0.07	0.4	64	0.6
17	0.42	0.35	0.07	0.4	64	0.6
18	0.47	0.35	0.12	0.8	64	1.2
19	1.14	0.35	0.79	4.9	64	7.6

Table I shows the results of a series of nitrogen determinations by the Kjeldahl method on the filtrates and washings of a number of determinations. The protein lost is calculated by multiplying the total nitrogen content of the filtrate less the non-protein nitrogen in the sample of plasma by 6.25. In 18 of the 19 observations, the percentage loss of protein was less than 3 per cent and in one 7.6 per cent. There is, therefore, no greater loss of protein in the filtrate than the limits of error of the method.

The possible inclusion of inorganic salts was tested by ignition after protein determination, using asbestos on Gooch crucibles instead of glass filters. The

final weights checked in each instance to within 1 per cent of the original weight of the filter.   The results of these determinations are shown in Table II.

The inclusion of fat is more difficult to detect and we have made no accurate measurements but have relied upon washing with alcohol, ether and hot absolute alcohol to completely remove fat.   Repeated washings with alcohol and ether do not change the weights of the filters and precipitates.

The results of this method are consistent and checks on duplicate determinations are easily obtained to within 0.3 gm. per cent.   Most of the observations to

TABLE II

Results of Ignition of the Filter after Protein Determination

Weight after 1st ignition	Weight after 2nd ignition	Weight after 3rd ignition following protein determination
gm.	*gm.*	*gm.*
18.8481	18.8472	18.8467
17.4932	17.4924	17.4921
17.8934	17.8924	17.8925
18.4737	18.4733	18.4729
17.6904	17.6904	17.6899
15.6526	15.6520	15.6517

TABLE III

Agreement of Duplicate Determinations on the Same Sample

No.	1	2	No.	1	2
1	5.54	5.45	7	7.24	7.30
2	5.54	5.25	8	6.41	6.47
3	5.82	5.76	9	7.21	7.09
4	6.49	6.25	10	5.04	5.01
5	5.44	5.47	11	5.20	5.14
6	5.74	5.64	12	6.89	6.99

be recorded here are based on single determinations but no great discrepancies were noted from day to day.   In a few instances, duplicate determinations were done and the agreement is shown in Table III.

In no case did duplicate determinations fail to agree except when they were done by different modifications of the method before the procedure described above was adopted.

The accuracy of the method for low serum protein values was demonstrated by measuring the protein content of a series of dilutions of normal plasma.   1 cc. was used in each case and was made up by adding to the amount of plasma shown

in the table, sufficient water to make 1 cc. The results are shown in Table IV and when the observed protein values are compared to the average of all the determinations, an error of less than 3 per cent is observed throughout the entire range except for the determination in which only 0.1 cc. of plasma was used.

TABLE IV

Accuracy of the Method over a Range of Plasma Protein from 0.6 to 7.0 Gm. Per Cent

Plasma cc.	Observed protein per cent	Average per cent	Difference per cent	Error per cent
0.1	0.65	0.69	0.04	5.8
0.2	1.34	1.38	0.04	2.9
0.3	2.11	2.07	0.04	1.9
0.4	2.75	2.76	0.01	0.4
0.5	3.54	3.45	0.09	2.6
0.6	4.18	4.14	0.04	1.0
0.7	4.74	4.83	0.09	1.9
0.8	5.55	5.52	0.03	0.5
0.9	6.32	6.21	0.11	1.8
1.0	7.04	6.90	0.14	2.0

TABLE V

Agreement between the Method Here Described and the Kjeldahl Method

No.	Protein, gravimetric per cent	Protein, Kjeldahl per cent	Difference per cent	Error (Kjeldahl as standard) per cent
1	7.04	7.19	0.15	2.1
2	5.42	5.40	0.02	0.4
3	5.62	5.90	0.28	4.7
4	5.64	5.60	0.04	0.7
5	5.79	5.50	0.29	5.3
6	6.37	5.80	0.57	9.8
7	5.55	5.52	0.03	0.5

There is no direct method, aside from the gravimetric, for measuring plasma protein and consequently there is no standard method against which the accuracy of another method may be tested. However, a few determinations were made by the method described and also by the Kjeldahl method, which showed an agreement to within about 5 per cent in all but one instance. The results are shown in Table V.

The method for plasma proteins here described has the advantages being direct, simple and of requiring very little time on the part the analyst, although the final results cannot be obtained in less n 1 or 2 days. It requires very small quantities of plasma and is urate to within 0.3 gm. per cent, which is quite adequate for cliniwork.
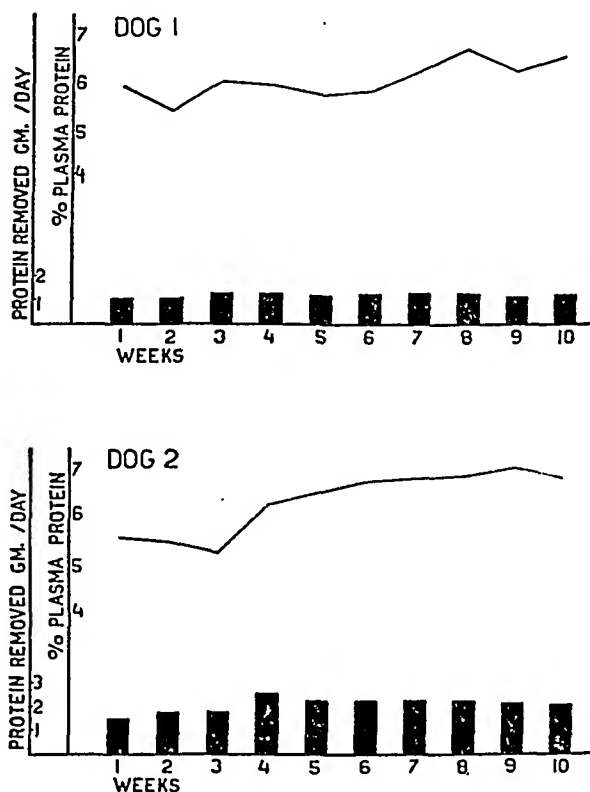

CHART 1. The effect on the plasma protein concentration of the daily loss of ein by repeated bleeding in dogs.

EXPERIMENTAL DATA

Experiments were carried out on five dogs weighing between 10 and kilos. The amounts of blood removed daily varied from 40 to 150 In Dogs 1 and 2, the hemoglobin did not fall appreciably, but in gs 3, 4 and 5 it fell steadily to about 50 per cent within the first eeks and remained at approximately this level throughout the eximent. This fall in hemoglobin resulted in a relative increase in

the amounts of plasma and hence of protein that could be removed as the experiment progressed. In each case the plasma protein fell slightly for the first 2 to 4 weeks and then increased to slightly more than the initial value by the end of the experiment. The results are shown graphically in Charts 1 and 2 and are summarized in Table VI. For the sake of brevity and to eliminate minor daily variations, the results are all expressed in weekly averages. Only total plasma proteins were measured.

All the dogs were on a liberal meat diet and no attempt was made to control it quantitatively, nor to determine the effect of diet on protein regeneration.
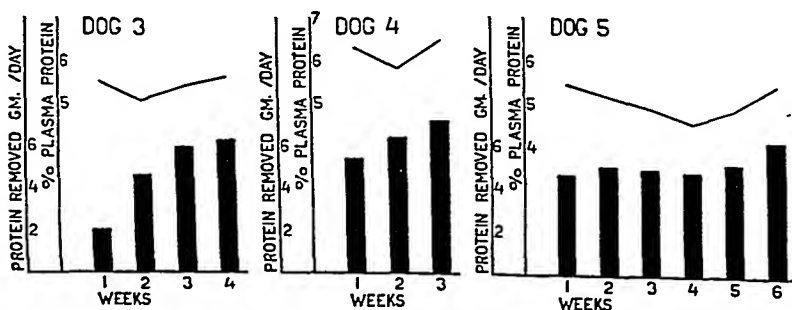

CHART 2. The effect on the plasma protein concentration of the daily loss of protein by repeated bleeding in dogs.

Dogs 1 and 2 remained in excellent condition throughout the experiment. The experiments on Dogs 3, 4 and 5 were terminated by the deaths of the animals. Autopsies were performed in each case but no cause of death was found beyond mild bronchopneumonia. Postmortem blood cultures taken in one case showed no growth. Experiments were started on three more dogs with the daily removal of 150, 200 and 250 cc. of blood but death occurred in all three before sufficient time had elapsed for the collection of significant data.

The rate of regeneration of plasma proteins in man has not been measured because of the danger of the removal of large amounts of blood proteins. In patients with kidney disease, large amounts of protein are often lost in the urine but this is usually accompanied by low plasma proteins, which may result either from the protein loss or

from failure of regeneration. We recently had a patient with cirrhosis of the liver who required frequent abdominal tapping and whose ascitic fluid contained considerable amounts of protein. This protein

TABLE VI

Effect on the Plasma Protein of the Daily Loss of Protein from Repeated Bleedings in Dogs

| Dog | Weight | Average daily removal | | Plasma protein | | Duration of experiment |
		Blood	Protein	Average 1st wk.	Average last wk.	
	kg.	*cc.*	*gm.*	*gm./kg.*	*per cent*	*per cent*	*wks.*
1	14.1	40	1.2	0.09	5.9	6.6	10
2	11.1	60	2.2	0.20	5.6	6.9	10
3	10.8	150	4.9	0.45	5.5	5.6	4
4	13.4	150	6.3	0.47	6.3	6.5	3
5	12.8	150	5.2	0.41	5.5	5.4	6

TABLE VII

Effect on the Plasma Protein of Loss of Protein in Ascitic Fluid

Date	Amount of fluid	Protein content	Protein lost	Protein lost	Plasma protein
	liters	*per cent*	*gm.*	*gm./day*	*per cent*
12/29/30	10.0	1.10	110	5.8	5.6
1/20/31	8.5	1.49	127	5.8	6.7
2/ 6/31	11.0	1.07	118	6.9	5.9
2/26/31	9.5	1.00	95	4.7	6.9
3/12/31	11.7	1.30	152	10.8	
3/28/31	11.7	1.37	160	10.0	6.1
4/11/31	12.5	1.22	152	10.9	5.9
4/28/31	14.0	1.60	224	13.2	5.5
5/16/31	13.1	1.63	214	11.9	
6/ 1/31	13.1	1.46	191	12.0	5.8
6/18/31	13.5	1.42	192	11.3	5.1
7/ 3/31	12.4	1.41	175	11.7	4.9
7/21/31	13.0	1.37	178	9.9	5.2

presumably was derived from the blood proteins and since there was no significant drop in the level of these, he was apparently able to regenerate sufficient to make up for the loss. A summary of the tappings and plasma proteins in this case is presented in Table VII.

At the end of the period of observation, this patient had produced 206 liters of ascitic fluid. Shortly after the last tapping a large umbilical hernia broke down allowing the constant escape of fluid and making further quantitative observations impossible. His urine was tested frequently and showed no significant abnormalities. His urinary protein excretion was never above the normal limits. The total period of observation was 204 days, during which he excreted a total of 2088 gm. of protein, an average of 10.2 gm. per day. His plasma proteins showed a good deal of variation during this time but there was no definite fall.

DISCUSSION

In all of the experiments described the level of the plasma proteins was maintained in spite of the daily removal of considerable quantities of protein. The duration of most of the experiments was sufficiently long to rule out the possibility of an emergency storage of protein for the purpose of maintaining this level, and it is assumed that regeneration was complete and sufficiently rapid to make up for the loss. There is a possibility that the concentration of the plasma proteins was maintained at the expense of a decrease in the total amount of circulating protein. In Dog 5, the plasma volume was measured by the method of Hooper, Smith, Belt and Whipple (10) at the beginning and at the end of the 4th week of the experiment. The initial plasma volume was 630 cc. and at the end of the 4th week it was 600 cc. These results agree within the limits of error of the method and do not indicate any decrease in the total amount of plasma.

In spite of the loss of widely varying amounts of protein, the constancy of the plasma protein levels in each individual dog is quite striking. In all cases except in Dog 2 in which it was slightly higher, the final level was almost identical with the initial one. The early fall with the subsequent rise to the original value suggest that the mechanism for regeneration requires some time for its complete development.

The regeneration of the plasma proteins can apparently be quite rapid and can be maintained over a long period of time. The daily regeneration in our experiments was of about the same magnitude as that observed by Smith, Belt and Whipple (2) but was maintained for

a period of 6 weeks in Dog 5 instead of from 2 to 7 days. That regeneration can be still more rapid is suggested by an experiment of Leiter (6) in which nearly a liter of blood was removed daily from a dog for 28 days and by one of Shelburne and Egloff (8) in which 13 liters of plasma were removed in the course of 37 days. In both of these experiments, the level of the blood protein dropped to extremely low values but, although the actual amount regenerated cannot be measured, it must have been tremendous.

These results indicate that the loss of considerable quantities of protein is not alone sufficient to produce lowering of the level of plasma protein and they suggest that in nephritis of the degenerative type, there may be an associated interference with the mechanism of regeneration. Linder, Lundsgaard and Van Slyke (11) conclude that protein loss is not sufficient to explain the low blood proteins in nephritis, although they observed low plasma proteins whenever the urinary protein excretion was above about 1 gm. for 24 hours. The daily loss of more than 10 gm. of protein over a period of 7 months in our case of cirrhosis had no appreciable effect on the concentration of plasma proteins.

SUMMARY

1. Experiments on five dogs are described consisting in the daily removal of blood plasma in amount from 25 to 100 cc. the red cells being returned to the circulation in Locke's solution. In no case was there a significant drop in plasma protein concentration.

2. A gravimetric method for the determination of total plasma protein is described.

3. A case is reported of cirrhosis of the liver in which over 10 gm. of protein daily was lost in the ascitic fluid during a period of 7 months without any lowering of plasma protein concentration.

4. The constancy of the plasma protein level and the adequacy of the mechanism of regeneration is pointed out.

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THE EFFECTS OF PARATHORMONE AND AMMONIUM CHLORIDE ON THE BONES OF RABBITS

By HENRY L. JAFFE, M.D., AARON BODANSKY, Ph.D., AND JOHN E. BLAIR, Ph.D.

(From the Laboratory Division of the Hospital for Joint Diseases, New York)

It has generally been concluded from the absence of effects or from the relatively slight effects upon serum calcium after parathormone injection in rabbits and guinea pigs that these animals are tolerant to parathormone (1–6). We have observed that young guinea pigs respond to the daily injection of relatively large doses of parathormone by decalcification and secondary fibrous transformation of the more rapidly growing bones or portions of bones (7). These effects occur in spite of the fact that the guinea pig is relatively resistant to parathormone, as measured by hypercalcemia, although with sufficiently large doses of parathormone both hypercalcemia and hyperphosphatemia may be induced (8). In young and adult guinea pigs a compensation is established during prolonged parathormone treatment, which enables them to tolerate repeated large doses without hypercalcemia, and which permits considerable repair of the bone lesions produced earlier in the treatment (8, 9).

We therefore studied histologically the bones of a rabbit treated with a single large dose of parathormone, and of a number of rabbits treated for long periods with parathormone alone, and with parathormone supplemented with calcium lactate or ammonium chloride.

Effect of a Single Large Dose of Parathormone

We found in a single test, 48 hours after an injection of 240 units of parathormone into a young rabbit (No. 301) weighing about 500 gm., definite hypercalcemia as well as hyperphosphatemia (serum calcium 17.2, serum phosphorus 9.3 mg. per 100 cc.). There was other evidence of extreme overdosage in a non-protein nitrogen value of 103 mg. per 100 cc. The animal was apparently moribund when

killed. The histological examination of the bones revealed unmis-takable resorption. Thus the hypercalcemia was obviously related in this case to calcium mobilization and removal. The resorption was very extensive and was associated with a fibrous reaction, particularly in the regions of active growth. The changes were similar in all respects to the changes observed in the bones of dogs and guinea pigs with acute hyperparathyroidism (7, 10).

Effect of Repeated Administration of Parathormone

A young rabbit (No. 1019), weighing 875 gm., received the following dosage of parathormone: 20 units daily for 7 days; 40 units daily for 49 days; 50 units daily for 14 days, and 60 units daily for 8 days. A total of 2080 units was given over a period of 78 days. The animal was killed on the 79th day, when it weighed 1530 gm.

Rabbits 1008 and 1003, young adult females, weighing 1225 and 1600 gm. respectively, received the following treatment: 20 units daily for 9 days; 40 units daily for 37 days; 50 units daily for 22 days; 60 units daily for 14 days, and 70 units daily for 8 days. A total of 4160 units of parathormone was given over a period of 90 days. On the 91st day the rabbits weighed 2075 and 2050 gm. respectively, and were killed to terminate the experiment.

In the gross, the bones of these three animals showed no evidence of softening, while on histological examination the lack of significant changes was also striking. Rabbit 1019 (the youngest when treatment was started) presented a very slight degree of scarring at the costo-chondral junctions and at the metaphyses, and there was slight sub-periosteal osteoclastic resorption in the regions of most active bone growth. The possible relation of these changes to the changes occurring in the course of normal bone transformation in young animals will be discussed.

Changes of about the same degree were present at the corresponding sites in Rabbit 1008, which was older and had received twice as much parathormone. No changes of active bone resorption were observed in the bones of Rabbit 1003, which was the oldest of the three (the only one in which no epiphyseal cartilage plates were present at autopsy). While osteoclastic resorption and fibrous replacement were not observed in this animal, there seemed to be a general thinning of both the cortex and of the spongy trabeculae at the epiphyseal ends of the long tubular bones.

Effect of Repeated Administration of Parathormone and Calcium Lactate

Rabbit 1007, a young adult female weighing 1425 gm., and Rabbit 632, a fully grown male weighing 2850 gm., received the following dosages of parathormone: 20 units daily for 9 days; 40 units daily for 11 days; 50 units daily for 48 days; 60 units daily for 14 days, and 70 units daily for 8 days. A total of 4470 units was given over a period of 90 days. During the same period calcium lactate was given in addition, in the form of a 10 per cent aqueous solution administered by stomach tube. The amounts were as follows: 1 gm. daily for 46 days; 1.5 gm. daily for 36 days; 2 gm. daily for 4 days, and 2.5 gm. daily for 4 days. On the 91st day the rabbits weighed 2400 and 2850 gm. respectively, and were killed.

No gross changes were observed in the soft tissues, and the bones cut with normal resistance. On histological examination no resorptive changes were observed in the bones. There was no apparent effect on the bones as a result of the administration of calcium lactate. Nor were there any areas of pathologic metastatic calcification in the soft tissues in spite of the administration of calcium lactate, which would tend to intensify the hypercalcemic effects of parathormone. In the dog and guinea pig the hypercalcemic effects of parathormone overdosage lead to the appearance of metastatic calcifications, especially in the kidneys, lungs, heart, etc.

Effect of Repeated Administration of Parathormone and Ammonium Chloride

Rabbit 1002, a young adult male weighing 1550 gm., and Rabbit 1009, a fully grown female weighing 2400 gm., received the following parathormone dosage: 20 units daily for 9 days; 40 units daily for 37 days; 50 units daily for 22 days, and 60 units daily for 22 days. A total of 4000 units was given over a period of 90 days. During this period ammonium chloride was given by stomach tube in a 10 per cent aqueous solution, in amounts as follows: 0.2 gm. daily for 19 days; 0.4 gm. daily for 14 days; 0.5 gm. daily for 49 days; 0.7 gm. daily for 4 days, and 0.8 gm. daily for 4 days. On the 91st day the rabbits weighed 1900 gm. and 2410 gm. respectively, and were killed. Two other rabbits were treated in similar fashion, and died probably as the result of ammonium chloride administration.

There was a complete absence of active resorption in the bones of these animals, in spite of the fact that parathormone and ammonium chloride employed separately or together produced marked decalcification and fibrous scarring in the bones of young dogs (11).

Rabbit 1001, a young adult female weighing 1800 gm., used as an